TRIMETHOPRIM RESISTANCE IN NORMAL FLORA FROM INDIA

STEVEN TAIT

THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF EDINBURGH
1993

ABSTRACT

Resistance to antibiotics is an increasing problem in the developing world. Resistance genes in the normal gut flora may act as a reservoir from which pathogens may acquire resistance. Trimethoprim is an important drug in the treatment of a wide range of infections but resistance has increased in recent years particularly in the developing world.

A survey was conducted in Vellore, south India, to determine the rate of carriage of antibiotic resistant enterobacteria in the normal gut flora of a sample of the population. Very high rates of resistance were encountered to the widely used antimicrobials, ampicillin, chloramphenicol and trimethoprim. The trimethoprim resistant strains were analyzed further, 35% were capable of plasmid-mediated transfer of trimethoprim resistance by different plasmid types, as identified by restriction endonuclease digestion. Resistance to a variety of other agents was co-transferred. Transferable trimethoprim resistance was shown, by DNA hybridization, to result from the presence of three different drug resistant dihydrofolate reductase (*dhfr*) genes. The *dhfr*V was present in 50% of transconjugants, associated with either the transposon Tn*21* or only with the integrase-like open reading frame (orf) of this transposon; 31% possessed the *dhfr*I, associated with the integrase orf of Tn*7* and 19% had *dhfr*IV.

The dihydrofolate reductase (DHFR) enzyme type IV has only ever been identified in this area of India, in 1984. The enzyme mediates only low level resistance, as measured by conventional tests, but it is inducible, a unique property in the DHFRs. It has persisted in this area despite a seemingly poor resistance mechanism. The DHFR typeIV enzymes characterized during this survey were also inducible. It was demonstrated that the induction mechanism will produce over 100-fold greater resistance if the cells are challenged prior to determination of resistance level. This mechanism was shown to be mediated by a lack of thymine and to be dependant on cell phase and density. A plasmid characterized in this study also mediated resistance to ampicillin as a result of the presence of the TEM-1 β-lactamase. This enzyme was also inducible, a property not observed before for this enzyme or for any other plasmid mediated β-lactamase in Gram-negative bacteria, producing increased resistance to the widely used amoxycillin-clavulanic acid combination. The induction of both enzymes was cross-reactive,

both trimethoprim and the β-lactam drugs induced both mechanisms. The metabolic impact of the β-lactams must be triggering the induction mechanism. This cannot, however, be by the same pathway as trimethoprim.

The high rates of carriage of normal gut flora resistant to antimicrobials are probably the result of selection by antimicrobials which are freely available without prescription combined with poor hygiene and sanitation. The evolution of such unusual induction mechanisms may have resulted from the prevailing low level of antimicrobials or frequent exposure to low levels of drug as a consequence of self-dosing. High rates of resistance in normal flora and the evolution of such resistance mechanisms is serious because of their impact on the development of resistance and in the testing for resistance, when such induction mechanisms may disguise true resistance levels.

The DNA sequence of the *dhfr*IV was also determined and was shown to be only distantly related to the chromosomal *dhfr* and other plasmid mediated *dhfr*s.

DECLARATION

The experiments and composition of this thesis are the work of the author unless otherwise stated.

"If we do not find anything pleasant, at least we shall find something new."

Françoise-Marie Voltaire

in "CANDIDE or OPTIMISM" (1759)

AKNOWLEDGEMENTS

Firstly, I must thank my supervisor, Professor Sebastian Amyes, for all of his help, encouragement, hard work and enthusiasm over these few years. I consider myself very fortunate to have had such a good boss. I am also indebted to my colleagues, Chris Thomson, Sara Nandivada and David Payne, who worked under difficult and often trying conditions in the heat and dust during the initial survey of resistance on which this work is based. I must also thank the staff at the Dept of Microbiology in the Christian Medical Hospital in Vellore for their part in this work. I am grateful, also, to Dr Hilary Young for her considerable help and useful discussions in the sequencing of the *dhfr*IV gene. I must also mention my colleagues in A.R.L. who have put up with me, although not without a bit of grumbling, and have made my job interesting to say the least. Finally, thankyou Clare for all of your patience and care.

This work is dedicated to my parents and grandparents.

PUBLICATIONS (in the duration of this thesis)

TAIT, S. (1989). The type S1 dihydrofolate reductase in *Staphylococcus aureus. J. Appl. Bacteriol.*, **67**,xiv.

AMYES S.G.B., TAIT, S. (1990). Trimethoprim resistance in *Staphylococci. J. Med. Microbiol.*, **31**, 4-7.

TAIT, S., *et al* (1990). Resistance to antibacterial drugs in commensal flora in south India. *J. Pharmacol.*, **42**, 64p.

AMYES, S.G.B., TAIT, S., THOMSON, C.J., *et al* (1992). The incidence of antibiotic resistance in aerobic faecal flora in south India. *J Antimicrob. Chemother.*, **29**, 415-25.

TAIT, S. (1993). Mobile Genetic Elements and Antibiotic Resistance. J Medical Microbiology. *J. Med. Microbiol.*, **38**,157-159.

THOMSON, C.J., TAIT, S., AMYES, S.G.B. (1993). Dissemination of the TEM-1 β-lactamase gene into disparate plasmids in faecal strains of *Eschericia coli* isolated in Vellore, south India. *J. Infect.*, in press.

PRESENTATIONS

TAIT, S., AMYES, S.G.B. (1991). Characterization of plasmids responsible for transferable trimethoprim resistance in commensal Enterobacteria. *Poster presentation at the 91st General Meeting of the American Society of Microbiology, Dallas, Texas.*

TAIT, S., SHANAHAN, P.M.A., THOMSON, C.J., AMYES, S.G.B. (1991). Antibiotic resistance in Scotland and India. *Poster presentation at the 5th European Congress of Clinical Microbiology, Oslo, Norway.*

TAIT, S., AMYES, S.G.B. (1992). The dihydrofolate reductase type IV. *Oral presentation at the Indian Congress of Nosocomial and Infectious Disease. Vellore India.*

TAIT, S., AMYES, S.G.B. (1993). Induction of the TEM-1 β-lactamase by Amoxycillin-Clavulanic acid. *Poster presentation at the 6th European Congress for Clinical Microbiology and Infectious Diseases. Seville, Spain.*

ABBREVIATIONS

A	Adenine
Ag	Amoxycillin/clavulanic acid combination, in 2:1 ratio
Ap	Ampicillin
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
cfu	Colony forming units
Cm	Chloramphenicol
Cp	Cephaloridine
dATP	2'deoxyriboadenosine triphosphate
dCTP	2'deoxyribocytidine triphosphate
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
dhfr	Dihydrofolate reductase gene
DM	Davis Mingioli minimal salts medium
DNA	Deoxyribonucleic acid
DSTA	Diagnostic sensitivity test agar
dTMP	2'deoxyribothymidine monophosphate
dUMP	2'deoxyribouridine monophosphate
EDTA	Ethylenediaminetetraacetic acid
*fol*A	Chromosomal dihydrofolate reductase gene
G	Guanine
ID_{50}	Dose giving 50% inhibition
IEF	Isoelectric focusing
In	Integron
ISTA	Isosensitest agar
kb	Kilobases
Km	Kanamycin
KVK	Kilvayatarrankuppam
MIC	Minimum inhibitory concentration
M_r	Relative molecular size
Mtx	Methotrexate
Na	Nalidixic acid
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
O.D.	Optical density
*p*ABA	Para-amino benzoic acid
PAGE	Polyacrylamide gel electrophoresis
R	Purine
r	Resistant
RNA	Ribonucleic acid
RUHSA	Rural unit for health and social administration

SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
Sm	Streptomycin
Sp	Spectinomycin
Su	Sulphamethoxazole
T	Thymine
Tc	Tetracycline
TD_{50}	Time at 45°C, in minutes, giving 50% inhibition
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
THF	Tetrahydrofolate
thy⁻	Thymine dependant auxotrophy
Tn	Transposon
Tp	Trimethoprim
Tris	Tris(hydroxymethyl)methylamide

The standard single and three letter abbreviations are used for the amino acids.

CONTENTS

CHAPTER 1

INTRODUCTION

Antimicrobial agents remain one of the few chemotherapeutic regimes that can effect a total cure of the disorder afflicting the patient. Trimethoprim, despite over 24 years of use, is still a potent drug in many infections. Its low cost has made trimethoprim an important agent in the treatment of a wide range of infections especially in the developing world. In many areas, however, particularly in the developing world, the use of trimethoprim is being compromised by high rates of resistance among enterobacteria (Levy *et al* 1987; Elwell and Fling 1989).

THE BEGINNING OF MODERN ANTIBACTERIAL CHEMOTHERAPY AND BACTERIAL RESISTANCE

1.1.1. The Early Development of Antibacterials

The development of trimethoprim (2,4-diamino-5-(3,4,5,-trimethoxybenzyl) pyrimidine) can be seen as leading directly from work done in the early years of antimicrobial chemotherapy by Woods on the sulphonamide antimetabolites (Woods 1940). The dye prontosil had been discovered earlier by Gerhard Domagt (1935) but had been shown to possess poor anti-bacterial activity. It was subsequently shown, by Tréfouel *et al*(1935) that the colourless metabolite of the drug, the sulphonamide component, was the active agent. Woods proposed that the sulphonamide produced, when the dye prontosil was metabolised, competed with *p*-aminobenzoic acid (PABA) in the formation of folic acid. Thus the site of action of the drug was identified (figure 1).

Exploitation of Fleming's famous discovery of penicillin (Fleming 1929) by Florey and Chain (Abraham 1983) switched the main thrust for the treatment of bacterial infections towards the true antibiotics produced by micro-organisms. There had

been previous reports of the inhibition of the growth of bacteria by moulds, and by other bacteria, by Pasteur and Joubert (1877) and as early as 1874 by William Roberts, who observed the inhibition of bacteria by *Penicillium glaucum*. These observations could not be exploited because the technology of the time was too limited. It was only after Fleming's report that the time was right for the exploitation of the antibiotic. Penicillin was purified and characterized by Florey and Chain (Abraham 1983) and production on a large scale was undertaken by large American chemical companies. This started the search for more and more antibiotics which were chemically modified and subsequently synthesized to improve efficacy and production.

1.1.2. The Development of Resistance

Very soon after the introduction of penicillin into clinical use, resistance mediated by penicillinases began to appear (Abraham and Chain 1940; Kirby 1944). This has been a continuing theme in antimicrobial chemotherapy. The introduction of greater and greater numbers and varieties of antibiotics has been met by the evolution of resistance mechanisms in the bacteria (Goldstein *et al* 1974). The development of such resistance mechanisms has not always been by stepwise mutations of the chromosome but has involved extra-chromosomal genetic material. The role of plasmids in the transfer of resistance between organisms was first identified by Akiba *et al* (1960) as cited by Watanabe (1963), and has since been shown to be relevant in most groups of antibiotics. More recently other molecular mechanisms have been described which are capable of mediating the movement of resistance genes between the replicons of host organisms, chromosomes and plasmids, revealing the enormous potential for the exchange of resistance genes between organisms and the development of resistance.

TRIMETHOPRIM AS AN ANTIBACTERIAL AGENT

1.2.1. The Action of Trimethoprim

The site of action of trimethoprim is the bacterial dihydrofolate reductase (DHFR)[5,6,7,8-tetrahydrofolate:$NADP^+$ oxido-reductase, EC 1.5.1.3], which is selectively inhibited, whilst the mammalian DHFR is not (Burchall and Hitchings 1965). The selective inhibition of the bacterial DHFR by trimethoprim in comparison to the mammalian enzyme, with 100,000-fold higher affinity for the drug, has been shown, by X-ray crystallography, to be the result of a much better "fit" of the trimethoprim molecule in the DHF binding site of the bacterial enzyme (Matthews *et al* 1986). The biochemical pathway upon which both trimethoprim and sulphonamides act is that which leads to the formation of tetrahydrofolate (THF), an essential co-factor in the synthesis of several amino acids, purines and pyrimidines (Burchall 1973; Hitchings 1973), particularly thymine (Amyes and Smith 1974a). Bacteria cannot take-up pre-formed folate molecules, therefore trimethoprim acts selectively against bacterial cells.

The sulphonamides inhibit the action of dihydropteroate synthetase, thus blocking the condensation of dihydropterate from *p*ABA and dihydropteridine. Dihyrofolate is then synthesised from dihydropteridine and dihydropteroate by the action of dihydrofolate synthetase. Dihydrofolate reductase then catalyses the reduction of dihydrofolate to tetrahydrofolate. Trimethoprim is a competitive inhibitor of dihydrofolate reductase, being an analogue of dihydrofolate (Brown 1971) (figure 1).

The metabolic effect on the cell of the inhibition of dihydrofolate reductase is wide ranging. Tetrahydrofolate serves as a carrier of one carbon units for the biosynthesis of methionine, formyl methionine tRNA and purines and is thus central to extensive metabolic processes. A product of THF, 5,10-methylene THF is involved in thymine biosynthesis. As well as donating the single carbon moiety, 5,10-methylene THF also donates 2 hydrogen atoms, this oxidation process regenerates DHF. (Figure 2). DHF is inactive in this metabolic cycle and must be reduced to the tetrahydrofolate state. Trimethoprim blocks the reduction to the "active" reduced form (Hitchings 1983).

Figure 1. The biosynthetic pathway of tetrahydrofolate inhibited by trimethoprim and the sulphonamides.

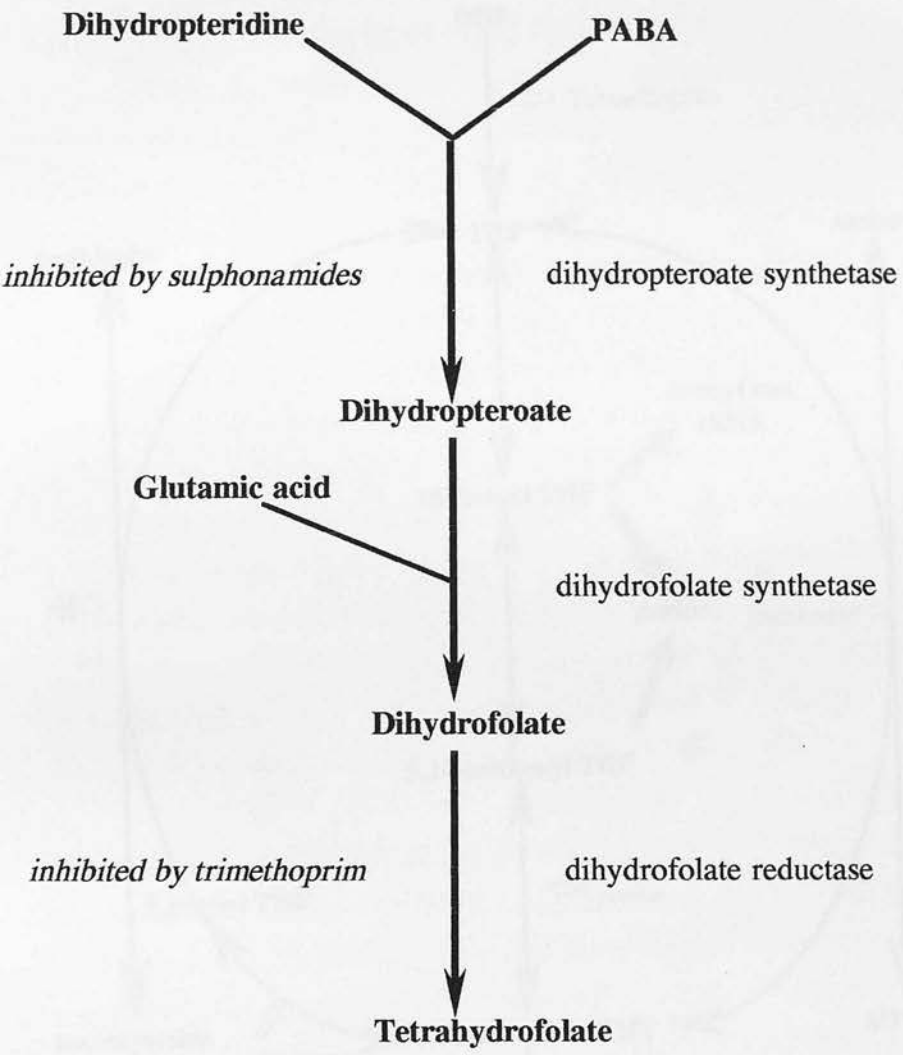
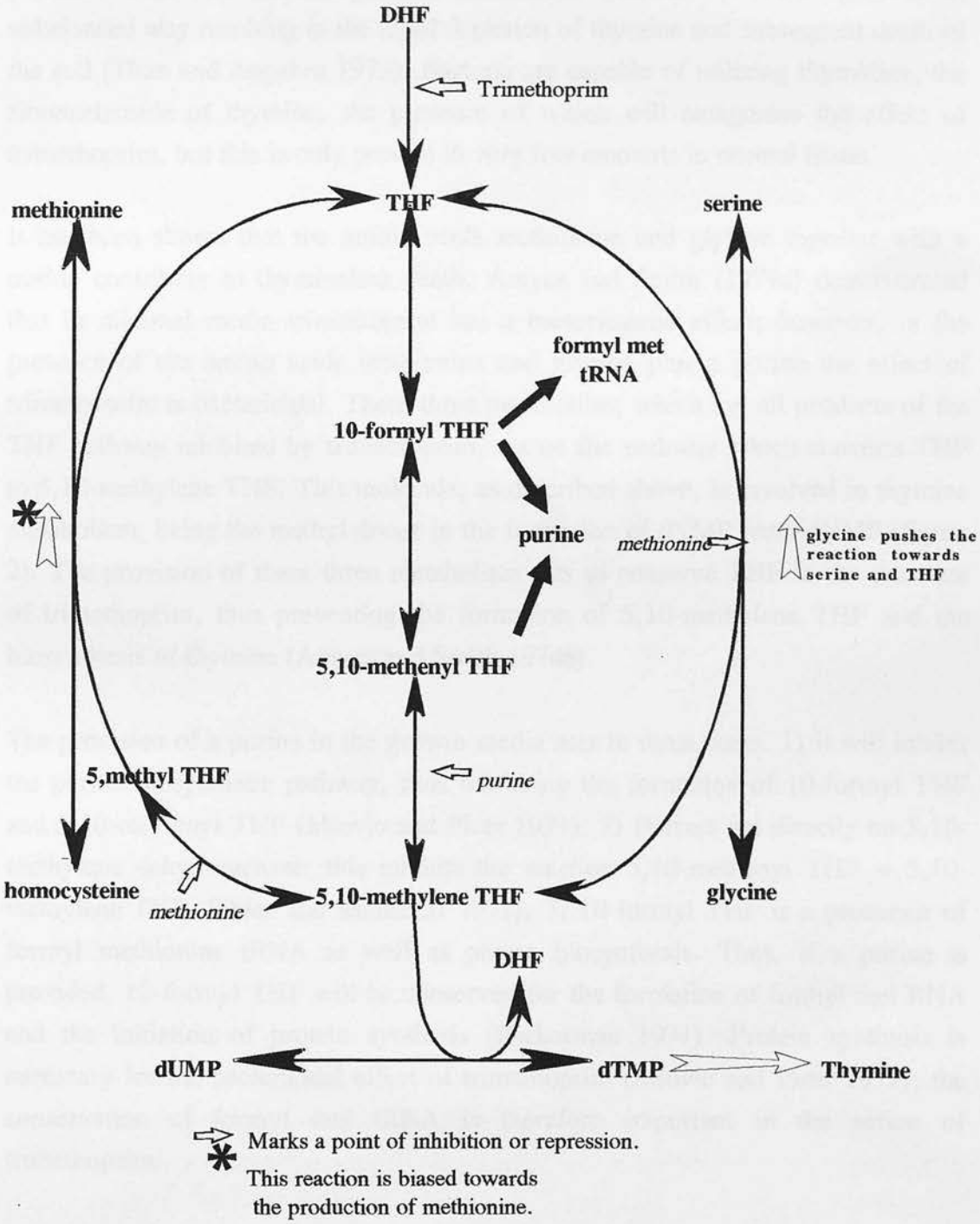


Figure 2. The pathway of 5,10-methylene THF formation from THF on which methionine, glycine and adenine act to produce thymine starvation in the presence of trimethoprim.



The bactericidal effect of trimethoprim is the result of thymine starvation (Then and Angehrn 1973). Bacteria are almost universally capable of utilizing exogenous purines and amino acids; however, they cannot take-up thymine. Thus in the presence of amino acids and purines, available in tissues, the bacteria grow in an unbalanced way resulting in the rapid depletion of thymine and subsequent death of the cell (Then and Angehrn 1973). Bacteria are capable of utilizing thymidine, the ribonucleoside of thymine, the presence of which will antagonise the effect of trimethoprim, but this is only present in very low amounts in normal tissue.

It has been shown that the amino acids methionine and glycine together with a purine contribute to thymineless death. Amyes and Smith (1974a) demonstrated that in minimal media trimethoprim has a bacteriostatic effect; however, in the presence of the amino acids methionine and glycine plus a purine the effect of trimethoprim is bactericidal. These three metabolites, which are all products of the THF pathway inhibited by trimethoprim, act on the pathway which converts THF to 5,10-methylene THF. This molecule, as described above, is involved in thymine metabolism, being the methyl donor in the formation of dTMP from dUMP (figure 2). The provision of these three metabolites acts to conserve THF in the presence of trimethoprim, thus preventing the formation of 5,10-methylene THF and the biosynthesis of thymine (Amyes and Smith 1974a).

The provision of a purine in the growth media acts in three ways. 1) It will inhibit the purine biosynthetic pathway, thus inhibiting the formation of 10-formyl THF and 5,10-methenyl THF (Miovic and Pizer 1971). 2) Purines act directly on 5,10-methylene dehydrogenase; this inhibits the reaction 5,10-methenyl THF $\Leftrightarrow$ 5,10-methylene THF (Sibler and Mansouri 1971). 3) 10-formyl THF is a precursor of formyl methionine tRNA as well as purine biosynthesis. Thus, if a purine is provided, 10-formyl THF will be conserved for the formation of formyl met RNA and the initiation of protein synthesis (Dickerman 1971). Protein synthesis is necessary for the bactericidal effect of trimethoprim (Miovic and Pizer 1971); the conservation of formyl met tRNA is therefore important in the action of trimethoprim.

The addition of glycine in the media will push the reversible serine $\Leftrightarrow$ glycine reaction, catalysed by serine hydroxymethyl transferase, in the direction of serine formation. This will act to conserve THF and deplete 5,10-methylene THF.

Methionine has two effects on 5,10-methylene THF formation. 1) Methionine has been shown to inhibit serine hydroxymethyl transferase; thus the formation of glycine and 5,10-methylene THF will be inhibited, as above, but in the presence of glycine this will have no further metabolic effect (Sibler and Mansouri 1971). 2) Methionine inhibits its own biosynthesis by inhibiting 5,10-methylene THF reductase. This enzyme catalyses the reaction 5-methyl THF ⇔ 5,10-methylene THF (Katzan and Buchanan 1965). The homocysteine ⇔ methionine equilibrium is strongly biased in favour of methionine, therefore the addition of methionine to the media does not greatly influence the flux of THF → 5,10-methylene THF (Amyes and Smith 1974a).

The addition of all three metabolites is required to produce the observed bactericidal effect (Amyes and Smith 1974a). They act in concert to prevent the formation of 5,10-methylene THF, and thus the formation of thymine, and to maintain protein synthesis. The organism undergoes thymineless death resembling that of a thymine requiring auxotroph in thymine deficient media (Amyes and Smith 1974a). Of course in the absence of trimethoprim the formation of THF from DHF must be sufficient to drive the pathway towards 5,10-methylene THF.

1.2.2. Trimethoprim in combination

Trimethoprim, first described in 1962 (Roth *et al* 1962), was formulated against a background of increasing resistance to the antibiotics. It was developed as an anti-metabolite acting on the same metabolic pathway as the sulphonamides (Figure 1) and was thus contrived as a potentiator of the sulphonamides (Bushby and Hitchings 1968). It was argued that the inhibition of DHFR by trimethoprim at a stage further along the pathway leading to folic acid synthesis would lead to synergy with the sulphonamides by "sequential blocking" (Hitchings 1969). It has also been argued that the sulphonamides themselves inhibit bacterial DHFR since sequential blocking of the same pathway could not produce an effect greater than that of the most active single inhibitor (Brown 1962). So trimethoprim and sulphonamide must act together on the DHFR in order to produce a synergistic effect (Poe 1976). It would seem likely, however, that the inhibition of dihydropteroate synthetase by the sulphonamides acts by limiting the supply of DHF, the substrate for dihydrofolate reductase; thus trimethoprim will be more effective, being in competition with lower levels of DHF (Hitchings 1973). This

results in increased bacteriostatic and bactericidal activity *in vitro*, and subsequent decrease in MIC (Bushby 1973). Besides the synergistic benefits in the use of both drugs together, it was hoped that the combination would act to delay the onset of resistance (Hitchings 1973).

1.2.3. The Use of Trimethoprim as a Single Agent

Despite the observed synergy of trimethoprim and the sulphonamides *in vitro* (Hitchings 1969; Bushby 1973), it has been argued that synergy will not occur *in vivo*. Sulphonamides act to lower the MIC of trimethoprim; thus synergy is seen only at trimethoprim concentrations below the MIC (Lacey 1979). Trimethoprim, because it inhibits the bacterial DHFR to such an extent, would need to be at sub-inhibitory levels and the sulphonamide at very high levels for synergy to be shown. Levels of trimethoprim found in tissue are usually much greater than the MIC for the infecting organism (Lacey 1979). The maximum synergistic effect is seen at ratios of trimethoprim to sulphonamide of 1:20 or 1:25 (Bergan and Brodwall 1972). This ratio is only observed in serum (Bergan and Brodwall 1972; Kaplan *et al* 1973); in other tissues trimethoprim is at much higher levels (Wilkinson and Reeves 1979) than this "ideal" ratio and will therefore be exerting the major if not all the effect of the combination.

It has been shown that there is no difference in the clinical efficacy of the combination over trimethoprim used as a single agent (Lacey *et al* 1980). *In vitro* models of urinary tract infection (UTI) have produced results in agreement with these findings. Levels of sulphonamide achievable in the bladder are not sufficiently high to contribute to the action of trimethoprim (Greenwood and O'Grady 1976; Greenwood 1979). Laboratory findings did, however, indicate that exposure to trimethoprim alone resulted in greater mutation to resistance when compared with exposure of bacteria to the combination (Darrel *et al* 1968). Results of clinical trials, however, reported no difference in cure rates or the emergence of resistance between trimethoprim, as a single agent, and the combination (Lacey *et al* 1980). A study conducted by Amyes, Doherty and Wannacot (1986) found no significant difference between the two regimes in the treatment of respiratory tract infections.

Some consensus has arisen that the combination has not delayed the development of resistance to either trimethoprim or to sulphonamide (Lacey 1982), as had originally been suggested. This probably resulted from the widespread resistance to sulphonamides before the introduction of trimethoprim. Many of these resistance genes were situated on promiscuous and widespread mobile genetic elements (Bissonnette and Roy 1992).

Trimethoprim as a single agent has been available in the UK since 1979. Towner and Slack (1986) reported an increase in the proportion of trimethoprim resistant sulphamethoxazole-sensitive *Enterobacteriaceae* from UTI after the introduction and increasing use of trimethoprim as a single agent. The authors also concluded that this had not resulted in a great increase in resistance to trimethoprim. In Finland trimethoprim has been available as a single agent since 1973 and increasingly high rates of resistance to trimethoprim have been reported associated with high levels of consumption of the drug (Huovinen *et al* 1986). General rates of resistance in Finland do not, however, seem to be higher than those reported in other western countries. Resistance in three Finnish cities was reported as between 14% and 19% in 1988 (Heikkilä *et al* 1990a). This compares to a rate of just below 20% in France (Goldstein and Acar 1990), where the combination is used extensively. Thus, the benefits in the use of trimethoprim alone, i.e. fewer adverse reactions, may outweigh the benefits of the use of the combination, which seem at best debatable *in vivo*. The argument may have swung away from the prevention of the onset of resistance, by the combination, to the suggestion that the combination may produce selective pressures that produce a linkage between the widespread sulphonamide resistance genes and trimethoprim resistance genes (Sundström *et al* 1988; Sundström and Sköld 1990).

THE EPIDEMIOLOGY OF TRIMETHOPRIM RESISTANCE

The introduction of trimethoprim has been followed by an increase in resistance found in clinical isolates as monitored in many regions (Goldstein and Acar 1990). However comparisons between studies are often difficult because of a number of factors such as patient groups, type of specimen and methodology may differ (Goldstein Papadopolou and Acar 1986; Amyes 1989) and there are local variations within areas (Elwell and Fling 1989). General trends can be seen, however, and sequential studies have provided useful information at a number of centres (Towner and Slack 1986; Towner 1982; Towner *et al* 1991; Heikkilä *et al* 1990a). With improved techniques more information is becoming available about the dissemination and mechanisms of resistance. The most striking feature in the epidemiology of trimethoprim resistance is the contrast between the developed world, where rates of resistance are generally low, and the developing world, where rates of resistance are considerably higher.

Enteric and diarrhoeal disease represents a major problem in many developing countries. Resistance in the bacterial pathogens causing such infections is a serious threat to the ability to combat such life threatening diseases (Farrar 1985). Resistance to trimethoprim is of particular importance, since organisms such as *Shigella, Salmonella* and *Vibrio* remained sensitive to trimethoprim whilst resistance has developed to ampicillin and chloramphenicol. This then represented a cheap and effective oral antimicrobial, with a long shelf life, with which to treat infections caused by bacteria which are increasingly resistant to the conventional therapy, ampicillin and chloramphenicol (Levy *et al* 1987; Shahid *et al* 1985).

1.3.1. Resistance to Trimethoprim in the Developed World

There have been numerous reports pointing to a general trend towards increasing rates of resistance to trimethoprim which seems to have stabilized in recent years (Goldstein and Acar 1990). From 1971-82, Gruneberg (1984) reported an increase in hospital isolates, in London, resistant to trimethoprim from 3.2 to 14.3%. Similar rates of resistance were seen in other studies centred in London hospitals, 13% in studies by both Brumfitt *et al* (1983) and Chirnside *et al* (1985). Similar figures were published for urinary tract isolates in Edinburgh over the period 1982-4 where overall resistance was 16.5% (Amyes, Doherty and Young 1986).

Hamilton-Miller and Purves (1986) reported an increase from 6 to 19% resistance to trimethoprim over the period 1981 to 1985. In a sequential study by Towner and Slack (1986) of Enterobacteria in UTI there was some increase in resistance to trimethoprim in *E. coli*, *Proteus* spp., *Klebsiellae* and *Enterobacter* in the community and hospitals in the Nottingham area. For example *E. coli* resistant to trimethoprim and sulphamethoxazole increased from 0.3% in 1978 to 11.3% in 1984 and 9.3% in 1985, in the community; and from 2.0% in 1974 to 14.0%and 12.0% in 1984 and 1985 respectively, in hospital isolates. As mentioned above the authors also noted an increase in the isolation of trimethoprim resistant sulphamethoxazole sensitive isolates concomitant with an increase in the use of trimethoprim as a single agent. Similar studies have been carried out in Finland with similar results. In Helsinki from 1978-1988 resistance in enterobacteria rose from 3 to 19%, and from 1980-1988 in Turku and Ronvaniemi the increase was 5 to 16% and 3% to 14% respectively (Heikkilä *et al* 1990a). However, Huovinen *et al* (1986) reported an increase of 8% to 35% during 1971 to 1984 associated with heavy use of trimethoprim in a Finnish hospital. In the USA resistance to trimethoprim is low, being reported at between 4% and 6% (Murray *et al* 1985).

There are also variations in patient populations; 40% resistance was reported in a Finnish geriatric hospital (Heikkilä *et al* 1990c), which compares to similar figures for geriatric wards in a British hospital of 50% in ward acquired infections but only 26% in infections acquired prior to admission (Bendall *et al* 1989). Amyes, Doherty and Young (1986) reported 64% of urinary bacteria resistant to trimethoprim from patients in a long-stay hospital in Edinburgh, compared to 16.5% in the general hospital population. These results probably reflect the nosocomial basis of the resistance in these situations and perhaps the high use of the drug to treat infections common in these patients.

Although there has been an increase in resistance to trimethoprim since the mid 1970s in western countries it is generally seen to be below 20%, besides exceptional patient populations.

1.3.2. Trimethoprim Resistance in Developing Countries

The situation in developing countries is quite different, with numerous reports of high rates of resistance (Goldstein, Papadopolou and Acar 1986). In a study of urinary pathogens in Vellore, south India, 64% of isolates were reported as being resistant to trimethoprim (Young *et al* 1986a). Similar figures were obtained in a study of trimethoprim resistance from Nigeria. 63.3% of urinary tract isolates were found to be resistant to trimethoprim, 93.3% of which had associated resistance to sulphamethoxazole (Lamikanra and Ndep 1989). In a three centre, study 44% resistance was reported in Santiago, Chile; 40% in Bangkok, Thailand, and 48% in Honduras (Murray *et al* 1985). A study in Chile reported 34% resistance occurring in Enterobacteria mostly from UTI, *before the introduction of the drug*, 78% of which was plasmid mediated (Urbina *et al* 1989). The authors speculated that other resistance determinants on the plasmids concerned were co-selecting for resistance to trimethoprim. In Tanzania 36% of *E. coli* were reported as being resistant to trimethoprim (Young and Amyes 1986b).

Murray and Matthewson (1990) demonstrated that resistant faecal flora was quickly acquired by visitors to developing countries; of 13 US students, previously clear of trimethoprim resistant faecal flora, 7 were shown to have acquired trimethoprim resistant faecal flora after a visit to Mexico, an area where resistance to trimethoprim is high (Murray and Matthewson 1990). Although this is only a very small study group it does demonstrate a route of transmission of resistance genes from third world areas to the developed world, especially given the increase in world travel.

Thus a contrast can be seen between the situation in developed and developing countries in the prevalence of resistance to trimethoprim. This is reflected in the increase in resistance to trimethoprim in those pathogens associated with enteric and diarrhoeal disease, a great problem in the developing world.

1.3.3. Trimethoprim Resistance in *Shigella*

Barada and Guerrant (1980) suggested trimethoprim as the drug of choice in the treatment of shigellosis resistant to ampicillin. Resistance to trimethoprim in *Shigella* had been virtually unknown until the late 1970s, when Bannatyne *et al*

(1980) reported that 3% of *Shigella* isolated in Ontario were resistant to trimethoprim. Other western countries showed similar increases. Between 1979 and 1983 Shigellae resistant to trimethoprim rose from 1.3% to 17% in England and Wales (Gross *et al* 1984). Spain in particular had a very high rate of resistance amongst Shigellae, 89% resistance was reported in 1983 (Palenque *et al* 1983). Recent reports from the Netherlands also show high rates of resistance in species of *Shigella*, resistance to trimethoprim amongst *S. sonnei* rose from 16% to 46%, in *S. flexneri* from 8 to 25%, in the period 1984-89 (Voogd *et al* 1992). In Finland it has been reported that resistance in shigellae that was only 3.0% had risen to 43.8% by 1988 (Heikkilä *et al* 1990b). A recent survey, again from Ontario demonstrated increasing resistance among shigellae, from the 3% previously reported to 26.7% in *S. boydii*, and 39.4% in *S. flexneri* (Harnett 1992). In the USA, however, general rates of resistance in shigellae are low, 7%, but higher rates were seen in people who had recently been abroad (Tauxe *et al* 1990).

Rates of resistance in shigellae reported in the developing world are considerably higher. In Bangladesh resistance rose rapidly after trimethoprim was introduced on a large scale in 1981 (Shahid *et al* 1985). Resistance among shigellae rose from 5% in 1979 to 83% in 1983 (Zaman *et al* 1983). There was also a concomitant switch in the dominant cause of shigellosis from *S. flexneri* to *S. dysenteria* type I (Shiga's bacillus) which had become resistant more rapidly than other species. This was a serious turn of events since *S. dysenteria* is a much more dangerous organism (Shahid *et al* 1985).

There have been similar reports from Zaire where therapy for shigellosis was switched from tetracycline to trimethoprim resulting in a rapid increase in resistance to 83% (Frost *et al* 1982). A similar situation was seen in India where 100% sensitivity to trimethoprim-sulphamethoxazole was described in 1981 amongst *S. dysenteria*; this, however, rose rapidly to 94% resistance in 1984 (Macaden and Bhat 1985). In Vellore, south India, 84% of *S. flexneri* and 88% of *S. shigae* were found to be resistant to trimethoprim, most as the result of transferable resistance (Jesudason *et al* 1989).

Trimethoprim resistance on a Navajo Indian reservation in the USA rose from 3% to 21% among shigellae. A correlation was also shown between those receiving antimicrobials and those infected by resistant organisms. Resistant faecal flora were

also shown in 28% of children who presented with symptoms but no single promiscuous plasmid could be identified as mediating resistance (Griffin *et al* 1989). The *in vivo* transfer of resistance to trimethoprim-sulphamethoxazole between *E. coli* and *Shigella* was also demonstrated in the same reservation. The index patient was shown to be a woman being treated prophylactically with the drug for a persistent urinary tract infection. An *E. coli* resistant to the combination was isolated from the urine and, subsequent to the development of shigellosis, a *Shigella* was isolated that was resistant to trimethoprim-sulphamethoxazole. Both organisms were found to harbour very similar resistance plasmids indicating that the *E. coli* was the source of resistance in the *Shigella* (Tauxe *et al* 1989).

1.3.4. Trimethoprim Resistance in *Salmonella*

Trimethoprim-sulphamethoxazole was shown to be as effective as ampicillin or chloramphenicol in the treatment of salmonellosis (Butler *et al* 1982), and, like *Shigellae*, resistance was not widespread, especially in *S. typhi*, before the late 1970's (Threlfall *et al* 1983). However, among *S. typhimurium* resistance to trimethoprim was 7%, largely as the result of the spread of a single phage type, 204c, (Threlfall *et al* 1980b), and the carriage of multi-resistant plasmids (Threlfall *et al* 1986), the acquisition of which was probably the result of the successive use of different antimicrobials to counter *S. typhimurium* in cattle (Threlfall *et al* 1986). 39% of cattle isolates and 4% of human isolates were phage type 204c, drug resistant strains.

The acquisition of drug resistance by *S.typhi* has been shown to be less likely (Paramasivan *et al* 1977). However, Tn*7* probably mediating DHFR type I (the most commonly isolated of the plasmid-encoded trimethoprim resistant DHFRs), has been encountered in *S. typhi* (Richards and Datta 1982). In Peru 14% of *S. typhi* were trimethoprim resistant (Taylor *et al* 1985); in Mexico a similar figure, 16%, was reported by Solorzano *et al* (1987).

1.3.5. Trimethoprim Resistance in *Vibrio*

Reports of trimethoprim resistance in Vibrio have been fairly infrequent. It was first demonstrated in a multi-resistant plasmid from *Vibrio cholerae*, in Dacca, Bangladesh (Threlfall, Rowe and Huq 1980). Again in the Indian subcontinent,

resistant *Vibrio cholerae* was reported in 1984 (Sunderan and Murthy 1984). More recently, transferable trimethoprim resistance in *V. cholerae* has been reported in Vellore, south India (Jesudason and John 1990).

The DHFR type II has been recorded as mediating trimethoprim resistance in *V. cholerae* in Thailand (Tabtieng *et al* 1989). It has been reported that plasmid mediated resistance to trimethoprim in *V. cholerae* is limited to the Inc 6-c incompatibility group (Goldstein, Gerbaud and Courvalin 1986).

Young and Amyes (1986b) demonstrated the presence of a plasmid, in 10 strains of V*ibrio cholerae* el tor Inaba from Tanzania, that encoded a resistance mechanism that was biochemically very much like the DHFR type Ia. The plasmid was thought to be derived from the plasmids of the *Enterobacteriaceae* that were prevalent in the area. The DHFR type I has also been shown integrated into the *V. cholerae* chromosome as part of Tn*7* (Goldstein, Gerbaud and Courvalin 1986).

TRIMETHOPRIM RESISTANCE IN NORMAL FAECAL FLORA

1.4.1. The Relevance of Resistance in Normal Faecal Flora to Disease States

Resistance in normal gut flora may be relevant to the occurrence of resistance in clinical isolates, for two reasons: 1) The gut flora themselves may cause an infection. 2) The gut flora may contribute to a gene pool of antibiotic resistance genes which may be acquired by pathogenic organisms (Hawkey 1986; Levy *et al* 1988). The transfer of R-factors *in vitro* is well documented, however, plasmid transfer *in vivo* in the gut is not (Anderson *et al* 1973). These authors demonstrated that, although they could not achieve transfer between two marked strains in the gut of volunteers in the absence of antibiotic, when therapeutic doses of antibiotic were administered transfer of the R-factors could be demonstrated. It seems likely, however, that under normal conditions R-plasmid bearing strains are eliminated from the gut after antimicrobial chemotherapy is ended, the resistant organisms do not persist in the gut, although it would seem that the organism itself is eliminated not the plasmid from the cell (Anderson 1974; Bennet and Linton 1986). Tauxe *et al* (1989) demonstrated the acquisition of a trimethoprim-sulphamethoxazole resistance plasmid by a *Shigella* from an *E. coli*, causing a urinary tract infection, which had previously been treated with trimethoprim.

Resistant strains may be passed from one person, receiving antimicrobials, to another who is not. Petroceilou *et al* (1977) reported that tetracycline resistant strains of *E. coli*, of the same serotype, could be isolated from a woman receiving long-term tetracycline therapy and her husband who received no antimicrobials. The same tetracycline resistance plasmid was also isolated in a different serotype of *E. coli*, that had not been carrying the plasmid at the onset of the study (Petroceilou *et al* 1977). This group had also previously demonstrated the transfer of R-factors in the gut of two healthy volunteers (Petroceilou *et al* 1976). In a follow-up study, cross-infection continued. Following long-term therapy with tetracycline, the woman was also treated with ampicillin. The tetracycline resistant organisms were eliminated to be replaced by ampicillin resistant *E. coli*. However, after all therapy had been stopped the tetracycline resistant organisms became dominant again. These strains persisted until they were replaced by tetracycline

sensitive *E. coli* of the same serotype as the resistant strain. This strain was also found to have a plasmid identical to the tetracycline resistance plasmid except for a 1Md insert which had stopped expression of tetracycline resistance (Petroceilou *et al* 1979). The curtailment of resistance expression confers an advantage on the cell when no challenge is present but the plasmid itself is maintained.

Thus it seems likely that normal flora carrying resistance genes can transfer these genes to other organisms and can spread between hosts, and that a background use of antimicrobials facilitates this process. However, doubt has been cast on the ability of normal faecal *E. coli* to cause infection, with the finding by Siitonen (1992) that such organisms isolated from healthy individuals did not possess the virulence factors found in organisms that cause infection. It was proposed that invasive organisms do not arise from the normal flora of the patient but are caused by organisms that first invade and colonize the gut and then infect other areas such as the urinary tract.

1.4.2. Surveys of Resistance in Normal Faecal Flora

In 1969, Datta reported 52% of admission samples at a London hospital contained organisms that were resistant to at least one antimicrobial, although resistance to any particular drug was not more than 38%, in the case of sulphamethoxazole. Linton *et al* (1970) showed 67% of children and 46% of adults harbouring resistant organisms. The rate in a rural sample was higher, at 63% of adults, presumably because of the use of antimicrobials in animal husbandry. The resistance was shown to be R-factor mediated in 61% of strains.

In a study conducted in Boston, 60% of isolates from normal faecal samples were resistant to one or more antimicrobial agents. Resistance to ampicillin was the highest; 40% of the samples contained *E. coli* resistant to ampicillin (Levy *et al* 1988). In a comparative study between Boston, Caracas, Peru, and Qin Pu, China, great differences were observed between the resistant organisms carried by children in Boston and children in the cities of the developing countries. 21 of 39 children had antimicrobial resistant *E. coli* in faecal samples, as opposed to 40 of 41 in Caracas and 51 of 53 in Qin Pu. In Boston, 1 of the 39 carried a trimethoprim resistant *E. coli*, but 25 of 41 in Caracas and 34 of 53 in Qin Pu had *E. coli* resistant to trimethoprim. None of the isolates from Boston were resistant to more

than six antibiotics but 6% in Caracas and 20% in Qin Pu were. These figures were directly reflected in the proportions of resistant organisms isolated from clinical specimens in these cities, demonstrating the link between resistance in normal and pathogenic flora (Lester *et al* 1990).

A study in the Sudan also showed high rates of resistance in the normal flora of children in developing countries. 96% carried ampicillin- and 77% trimethoprim-resistant *E. coli*; 19 of 40 tested possessed R-factors mediating resistance (Shears *et al* 1988).

In a study of students from the Netherlands, 25% of normal faecal samples contained *E. coli* resistant to trimethoprim, 86% to sulphamethoxazole and 76% to ampicillin (Bonten *et al* 1990). In a recent study the same group presented similar high figures for resistance in two industrial centres in the Netherlands; 89% resistance to ampicillin and 28% resistance to trimethoprim (Bonten *et al* 1992). These figures are high and high rates of resistance amongst pathogenic organisms have also been reported in the Netherlands. Resistance to ampicillin in *Shigella flexneri* was reported at 53% and resistance to trimethoprim in *S. sonnei* at 46% (Voogd *et al* 1992).

Under circumstances where faecal contamination is high, because of poor hygiene, spread of resistant organisms is high, as reported in the study of a children's day-care centre by Reves *et al* (1990). Twenty eight percent of samples contained trimethoprim-resistant *E. coli,* and 65% contained ampicillin-resistant *E. coli.* These centres, it was suggested, could represent important foci for the dissemination of resistant organisms (Reves *et al* 1987). It has subsequently been demonstrated that members of the families of children harbouring resistant strains also have trimethoprim resistant strains in their normal flora (Fornasini *et al* 1992).

High rates of resistance in normal flora will be reflected in resistance in clinical isolates since these arise directly, or may acquire resistance factors from, normal flora (Lester *et al* 1990). Thus, monitoring of resistance in normal flora may give important indications of resistance in clinical isolates (Bonten *et al* 1992).

1.4.3. Conditions Supporting the Development of Resistance

Life threatening enteric pathogens are capable of rapid acquisition of resistance. This represents a considerable problem in developing countries where such infections are far more common and much more dangerous. The emergence and spread of resistance is supported by overuse and misuse of antimicrobial drugs (Griffin *et al* 1989; Levy *et al* 1987). The causes of such misuse are complex and many. Some of the factors involved are: Poor diagnosis through lack of resources and education resulting in poor selection of antimicrobial agents in the treatment of infections; misconceptions of the efficacy of antimicrobials, there is a pervading attitude that antimicrobials are "wonder-drugs" that can cure many ailments; promotion by drug companies can often be misleading, forceful and very persuasive. These factors lead to a "shot-gun" approach to the use of antimicrobials with considerable sub-optimal use. Restrictions on the purchase of antimicrobials, whether *de facto* or *de jure*, are fewer in developing countries which results in over the counter purchase and self-dosing (Levy *et al* 1987).

The spread of resistant organisms is also assisted by poor hygiene, over-crowding, poor general health and living conditions (Griffin *et al* 1989; Levy *et al* 1987). This can result in the constant circulation of resistant organisms from those who are taking antimicrobials.

In some areas, reduction in drug use, switch to support therapy for mild infections and better hygiene may be possible (Griffin *et al* 1989). However, the problems of third world regions are considerable. The appropriate use of antimicrobials must not be discouraged since the need is great in regions where bacterial infections are widespread and much more dangerous (Levy *et al* 1987).

MECHANISMS OF RESISTANCE TO TRIMETHOPRIM

The novel and synthetic nature of trimethoprim has presented an ideal opportunity to observe the development of resistance to an antibacterial from day zero (Amyes and Towner 1990). The antibiotics such as penicillin are derived from natural products, thus resistance to such compounds will be extant in the bacterial gene pool. There was no such background in the development of resistance to trimethoprim since it was a totally novel agent.

The bacteria have not disappointed us in their response to trimethoprim. The target site of the drug was novel and the subsequent evolution of the organisms has been as novel. Five responses conferring protection from the effects of trimethoprim on the cell have been characterized.

1. Thymine auxotrophy.
2. Impermeability.
3. Mutation in the chromosomal DHFR structural gene.
4. Hyperproduction of chromosomal DHFR.
5. Production of a second DHFR with low affinity for the drug.

1.5.1. Thymine Auxotrophy

Trimethoprim was used to produce thymine requiring auxotrophs *in vitro* by Stacey and Simson (1965) and Bertino and Stacey (1966) by growing organisms in the presence of trimethoprim and thymidine. These mutants are highly resistant to trimethoprim (Amyes and Smith 1975). Thymidylate synthetase is non-functional, thymine is acquired directly from the medium. This effectively "shortcircuits" the THF pathway, the action of trimethoprim can no longer produce thymineless death. Obviously, this is only an effective resistance mechanism as long as thymine is present in the medium. Thymine is not usually present in normal tissue and therefore the occurrence of such mutants in this case is not possible (Maskell *et al* 1978). There have, however, been several reports of such mutations arising during trimethoprim therapy (Okubadejo and Maskell 1973; Hayek and Netherway 1976). Acar and Goldstein (1982) reported that 1.5% of trimethoprim resistant strains were thymine requiring auxotrophs. For these mutants to arise there must be an underlying source of thymine or a derivative which supports the growth of the

auxotrophs. Dead pus cells or bacteria in renal calculi are possible sources of thymine (Maskell *et al* 1976).

1.5.2. Impermeability

Pseudomonas aeruginosa is intrinsically resistant to trimethoprim despite possessing a chromosomal DHFR which is sensitive to the drug. It has, therefore, been argued that there must be a permeability barrier preventing trimethoprim from entering the cell (Burchall *et al* 1982). Resistance to trimethoprim as a result of reduced penetration into *P. aeruginosa* has been reported (Werner and Goeth 1984) but the relevance of impermeability to resistance to trimethoprim has been questioned (Scudamore and Golder 1989). Permeability mutants of strains normally sensitive to trimethoprim have also been reported. Mutants of *Klebsiella pneumoniae* were isolated that possessed cross-resistance to nalidixic acid, chloramphenicol and trimethoprim following exposure to any of the drugs; it was speculated that the cross-resistance could be the result of a permeability barrier (Smith 1976). This phenomenon has also been reported in *Serratia* and *Enterobacter* but has not been demonstrated in *Escherichia coli* or *Proteus* (Acar and Goldstein 1982). On examination of the outer-membrane proteins, these strains of *Serratia* and *Enterobacter* were found to be deficient in proteins corresponding to porins. There was also a concomitant reduction in the uptake of [^{3}H]-glucose and [^{14}C]-chloramphenicol indicating a change in the permeability of the cell wall (Gutmann *et al* 1985). It appears likely that this is a true impermeability resistance mechanism produced *in vitro*. Thirty such mutants were also isolated from patients (Gutmann *et al* 1985). However, the epidemiological significance of such a mechanism is a matter of debate; impermeability is often sited when no other mechanism can be identified to account for the observed resistance (Hamilton-Miller 1979).

1.5.3. Alteration in the Chromosomal Dihydrofolate Reductase

Mutations in the DHFR structural gene, that result in reduced affinity of the enzyme for the drug rendering cells more resistant, as measured by higher DHF K_i values in the presence of trimethoprim, can be produced by chemical mutagenesis or the selection of spontaneous mutants, *in vitro* (Smith *et al* 1982). These mutations in the structural gene are often accompanied by changes in the regulation of production of the enzyme resulting in higher levels of DHFR with a reduced sensitivity (Sheldon 1977). Smith *et al* (1982) found that mutations occurred in two specific amino acids at a highly conserved region associated with DHF binding (Freisheim *et al* 1978).

Although these mutations have been studied in detail in laboratory mutants their clinical significance has not been widely investigated. Thirteen highly resistant strains of *P. cepacia* were shown to have an altered DHFR resulting in decreased drug sensitivity. However, this could not solely account for the high level resistance observed and permeability or hyper-production may have been involved (Burns *et al* 1989). Degroot *et al* (1991) have reported decreased chromosomal DHFR combined with hyper-production of the enzyme as being responsible for trimethoprim resistance amongst isolates of *Haemophilus influenzae*. A survey of 133 strains reported chromosomal DHFRs with reduced sensitivity in *Enterobacter* spp. and *Klebsiella aerogenes* (Grey *et al* 1979). There are no methods for the rapid detection and analysis of altered chromosomal DHFR. The purification and biochemical analysis of enzyme samples is extremely time-consuming; the performance of any meaningful survey would thus be a practical impossibility.

1.5.4. Hyper-production of Dihydrofolate Reductase

Resistance to trimethoprim by the over-production of the chromosomal DHFR is rare amongst clinical isolates (Elwell and Fling 1989). The constitutive over-production of DHFR would probably result in an imbalance which would seriously disrupt the metabolism of the cell (Koch 1981). Such mutations have, however, been produced *in vitro*. Sheldon (1977) produced three mutants that were resistant to trimethoprim having DHFRs that were hyper-produced, 10-fold, and had reduced affinity for trimethoprim. It has been suggested that DHFR is responsible for its own regulation, by autogenous control of the promoter, because alterations

in the structural gene can lead to over-production, suggesting that the DHFR must be acting as an operator on its own promoter (Goldberger 1974). This seems an unlikely scenario since the regulation of DHFR has been shown to be cis-acting and it is improbable that DHFR is an autogenous cis-acting regulator (Sheldon and Brenner 1976). Sheldon (1977) proposed alternative explanations; mutations for increased DHFR production and reduced affinity for trimethoprim occurred at 10^{-8} or 10^{-9} suggesting a single event so, unless DHFR is a cis-acting regulator, the mutation must act to increase the efficiency of initiation of translation.

Tennhammar-Ekman *et al* (1986) reported resistance in a clinical strain of *E. coli* as the result of over-expression of chromosomal DHFR. The enzyme was over-produced 200-fold and also had reduced affinity for trimethoprim. The production of the enzyme was also increased by the addition of trimethoprim to the medium; higher levels of enzyme were induced three minutes after the addition of the drug to exponentially growing cells at a cell density of $2x10^8$ cells/ml. This process was dependent on protein synthesis and was not produced by thymine starvation with the addition of the uridine analogue 5-fluorouracil; although this was not tested directly by the use of thymine auxotrophy or the addition of thymine to the medium to antagonise the effect of trimethoprim. Over-production of the DHFR has subsequently been shown to be the result of more efficient transcription and translation of the *fol* gene through 1) mutations in the ribosomal binding region leading to increased homology with the 16S ribosomal subunit, 2) lengthening the distance between this region and the start codon and 3) mutations in the promoter region. These changes all result in increased transcription and translation (Flensburg and Sköld 1987). Smith *et al* (1982) found similar changes in constitutive "promoter-up" mutations produced in the laboratory. However, these findings do not explain the induction by trimethoprim of further increases in the production of DHFR. No mechanism has been suggested for the induction of the chromosomal enzyme (Tennhammar-Ekman *et al* 1986).

1.5.5. Resistance Mediated by the Presence of a Second Dihydrofolate Reductase with Reduced Affinity for Trimethoprim

The most important mechanism of resistance is that mediated by the production of a second insensitive DHFR, thus by-passing the block imposed by the action of trimethoprim on the sensitive enzyme (Amyes and Smith 1974b; Sköld and Widh 1974).

Table 1. The biochemical properties of the plasmid mediated DHFRs.

Enzyme	Tp ID_{50} μM	Mtx ID_{50} μM	TD_{50}	DHF K_m μM	Tp K_i μM	Size Kd.	MIC mg/L
Ia	57.0	4.4	0.5	5.6	7.4	35	>1000
Ib	32.0	2.8	1.2	11.0	41	24.5	>1000
IIa	70000.0	1100.0	>12	4.6	6100	35	>1000
IIb	80000.0	750.0	>12	8.3	150	35	>1000
IIc	20000.0	1000.0	>12	4.2	400	34	>1000
IIIa	2.0	0.2	>12	0.4	0.019	16.9	64
IIIb	2.0	0.2	>12	9.5	0.4	17	128
IIIc	3.0	0.007	8	3.1	0.5	22	256
IV	0.2	0.02	>12	37.0	0.063	46.7	10
V	20.0	2.3	*	15.4	3.2	§5	>1000
VI	200.0	7.25	0.4	31.2	75.0	10	>1000
VII	30.0	3.0	1.5	20.0	7.0	11.5	>1000
VIII	-	-	-	-	-	-	>1000
IX	0.01	-	-	-	-	19.86	250
X	-	-	-	-	-	21.2	500
XII	-	-	-	-	-	-	>2000
S1	50.0	0.002	>12	10.8	11.6	19.7	>1000

* Dependent on protein concentration.

§ Apparent molecular weight by Sephadex gel filtration.

Plasmid mediated resistance to trimethoprim was first described in 1972 by Fleming *et al.* It has subsequently been shown to be mediated by a variety of mobile genetic elements (Sundström *et al 1988*: Bissonnette and Roy 1992). A number of plasmid mediated DHFRs, insensitive to the action of trimethoprim, have been described and classified, initially based on their biochemical properties (Amyes 1989; Huovinen 1987). Those enzymes recently described have been

classified solely on their DNA sequences and hybridisation with known gene probes, nothing being known of their biochemical properties (Amyes *et al* 1992).

1.5.5a. The Type I Dihydrofolate Reductase

Sköld and Widh (1974) described the by-pass resistance mechanism in relation to the enzyme produced by the plasmid R483. The enzyme was subsequently designated the DHFR type I (Pattishall *et al* 1977). The enzyme was found to be moderately resistant to trimethoprim; Tp ID_{50} (the concentration of drug required to reduce activity by 50%) was shown to be 57μM. The enzyme is, however, produced in 10-fold greater quantities than the chromosomal enzyme, rendering the cell resistant to high levels of drug. It was also shown to be heat labile (Pattishall *et al* 1977). The enzyme has a dimeric subunit structure, each subunit having a molecular weight of 18,000 (table 1). This in contrast to the monomeric structure of the chromosomal enzyme, although this has a molecular weight of 18,000 equivalent to that of the single subunit of the type I (Fling and Elwell 1980). When the DNA sequence of the subunit is aligned with that of the chromosomal enzyme, homology can be seen between the two enzymes, greatest homology occurring around the substrate binding site (Fling and Richards 1983). Recent analysis puts the similarity between the two enzymes at 57% with 37% identity (Singh *et al* 1992). Such homology points to a common ancestor or the resistant enzyme has been introduced from a species very close to *E. coli* (Fling and Richards 1983).

The DHFR type I is the most commonly isolated of the trimethoprim resistant DHFRs. It is located on the transposon Tn*7* (Barth *et al* 1976), although it has also been isolated in association with Tn*21* (Sundström and Sköld 1990). The transposon Tn*7* inserts into a specific site in the *E. coli* chromosome, the Tn*7* attachment site (attTn*7*), with very high frequency and in one orientation, although it will also transpose at lower frequency to other sites in the chromosome and other replicons (Lichtenstein and Brenner 1981; 1982). Thus, Tn*7* and DHFR type I have a tendency to become fixed in the chromosome of *E. coli*, integrated at the attTn*7* site (Heikkilä *et al* 1991). So the DHFR I has become more frequently identified in the chromosome of clinical isolates (Towner *et al* 1980; Kraft *et al* 1986; Steen and Sköld 1985 Heikkilä *et al* 1991).

The DHFR type I is the dominant DHFR found in resistant isolates, especially in the industrialised world. Towner *et al* (1991) reported 86.4% of trimethoprim resistant *E. coli* from Nottingham harboured the DHFR type I in 1978-83; this, however, dropped to 62.3% in 1987-88. In a survey in Finland, 73% of resistant isolates were found to have the DHFR type I, mostly integrated in the host chromosome (Heikkilä *et al* 1991). In a wider survey of trimethoprim resistance in Finland, although it was less frequently identified, the DHFR type I was still the dominant resistance mechanism, and increased from 51% of resistant isolates in 1984 to 63 % in 1987 (Heikkilä *et al* 1990a). A recent report of a survey in Taiwan reported 45.4% of resistance to trimethoprim mediated by the DHFR type I (Chang *et al* 1992). Of trimethoprim resistant *Shigella* spp. in Finland, 85% of resistance was mediated by the DHFR type I, the enzyme being present in 35% of all *Shigella* spp. (Heikkilä et al 1990b). The gene has also been detected in *Shigella dysenteriae*, both on plasmids and in the chromosome, in isolates from various parts of south east Asia (Haider *et al* 1990). Similarly Tn*7* has been shown to mediate trimethoprim resistance in *Salmonella typhi* (Richards and Datta 1982). Tn*7*-like transposons have also been implicated in trimethoprim resistance in *Vibrio* spp., both integrated in the chromosome (Goldstein *et al* 1986) and in plasmid mediated resistance in *Vibrio cholerae* el tor inaba (Young and Amyes 1986).

The DHFR type I has also been recently reported as a "gene-cassette" inserted into Tn*21* and thus linked to the sulphonamide resistance gene which is associated with this element (Sundström and Sköld 1990). It has been reported in this new location in 6 of 75 isolates in Finland (Heikkilä *et al* 1991). This gives a new twist in trimethoprim resistance conferred by the type I DHFR, provided by "gene-cassette" structures and integrons.

A DHFR with similar but distinct properties to the DHFR type I (table 1), has also been characterized and designated as the type Ib DHFR (Young and Amyes 1985). It was originally purified from a strain of *E. coli* isolated in Edinburgh but until recently no DNA probe was available so little is known of its epidemiology (Qumsieh and Young 1991).

1.5.5b. The Type II Dihydrofolate Reductases

The type II DHFRs are a group of three related enzymes showing significant homology at the DNA level (Flensburg and Steen 1986; Stone and Smith 1979; Swift *et al* 1981) and very similar biochemical properties (Pattishall *et al* 1977), which must share a common origin. The type II enzyme of the plasmid R388 was the first identified instance of trimethoprim resistance mediated by production of a second DHFR insensitive to the drug (Amyes and Smith 1974b); the enzyme has since been designated as the type IIb. The type IIa, of plasmid R67, was shown to be almost completely insensitive to the action of trimethoprim, the ID_{50} being 70mM compared to 57μm for the DHFR type I (table 1) (Pattishall *et al* 1977). These enzymes all have molecular weights of 35,000, having a tetrameric subunit structure, each subunit being M_r 8,000 (Joyner *et al* 1984). Sequence analysis has confirmed these findings and shown that the subunits are homogeneous (Flensburg and Steen 1986; Stone and Smith 1979; Swift *et al* 1981). Sequence analysis has also shown the types IIa, b, and c to be completely unrelated to the other DHFRs. This would account for the very high resistance of the enzymes to trimethoprim and has provoked speculation that these enzymes are oxido-reductase enzymes that have assumed DHFR function (Elwell and Fling 1989).

The type II DNA probe was initially generated from R67 providing an 850bp probe (Mayer *et al* 1985). However, this probe contains a portion of the widespread transposon Tn*21* and produces frequent false positives (Towner 1990). An alternative is the 280bp fragment in pWZ280 derived from R388 (Zolg and Hangii 1978). The probes can be used in the detection of any of the three type II DHFRs because of the high degree of homology that they share.

The prevalence of the type II DHFRs in Europe is low; 1.5% of trimethoprim resistant *E. coli* (Towner *et al* 1991), and only 3 isolates of 1300 resistant *Enterobacteriaceae* in Finland (Heikkilä *et al* 1990), carried a type II enzyme. A report from Greece, however, showed the type II DHFRs to be the dominant form of trimethoprim resistant DHFRs in species other than *E. coli*, i.e. *Klebsiella, Enterobacter and Serratia* spp., although the type II was also reported in a small number of *E.coli* (Tsakris *et al* 1993). This seemed to be the result of the spread of related plasmids amongst these species (Tsakris *et al* 1992); the authors speculated that the plasmids carrying the two different genes had different evolutionary origins

(Tsakris *et al* 1993). Both of these surveys employed the small probe fragment and could be considered reliable. Other reports outside Europe have also reported high prevalence of type II DHFRs. These have, however, employed the large type II probe and false positives may have been counted. Thirty two percent of trimethoprim resistant *Shigella* spp. and 50% of enterotoxigenic *E. coli* harboured type II DHFRs, compared to 9% and 14% carrying the DHFR type I (Chatkaemorakot *et al* 1987). Sixty per cent of trimethoprim resistance in Chile (Murray *et al* 1985), and 26% in a worldwide selection of enterobacteria (Fling *et al* 1982) was type II mediated. Tabtieng *et al* (1989) reported trimethoprim resistance in an outbreak of *Vibrio cholera* as the result of two plasmids carrying type II dihydrofolate reductase.

The type II DHFRs, despite the presence of the IIc on a transposon (Shapiro and Sporn 1977), seem to have a localized distribution and occur only as plasmid mediated resistance (Tsakris *et al* 1992; Papadopolou *et al* 1986).

1.5.5c. The Type III Dihydrofolate Reductases

The type III DHFR was first identified by Fling *et al* (1982), after a small mobilisable plasmid conferring low level trimethoprim resistance in *Salmonella typhimurium* failed to hybridize with either the type I or II probes. The enzyme carried by this plasmid was shown to be sensitive to low levels of trimethoprim, reflected in the ID_{50} 1.5μM. High affinity for the substrate, however, shown in the low DHF K_m (table 1), confers a moderate level of resistance on the cell. The minimum inhibitory concentration is 64mg/L. It is a monomeric protein of M_r 16,900; this structure, size and high level of DNA homology, 51% identity, reflects a close relationship to the chromosomal DHFR of *E. coli* (Fling *et al* 1988).

Despite being originally isolated on a broad host range plasmid of incompatibility group Inc Q (Hedges 1987), and speculation that this would lead to rapid spread of the enzyme (Joyner *et al* 1984), reports of the type III have remained few. Its presence in the UK has been confirmed (Thomson *et al* 1990) and there has been a report of increasingly frequent detection of the type III gene in Nottingham, from 1.2% in 1978 to 20.3% in 1987-88 in trimethoprim resistant *E. coli* (Towner *et al* 1991). The low frequency of detection of the type III DHFR may be as a result of

the less frequent testing of low level trimethoprim resistant strains for transferability or the presence of insensitive DHFRs. It is often assumed that resistance of this low level is chromosomal in origin. It has been noted that the presence of low level transferable resistance to trimethoprim means that it is important to test such isolates for the presence of transferable trimethoprim resistance (Towner and Pinn 1981).

More recently two enzymes have been identified in the US. that have been designated as the types IIIb and IIIc DHFRs because of their close biochemical similarities to the original type III, the IIIa. Both of the enzymes were identified in plasmids from *Shigella sonnei* outbreaks (Barg *et al* 1990). Moderate levels of resistance is conferred by these enzymes which have only slightly raised insensitivity to trimethoprim and very similar ID_{50} figures (2.0μM and 3.0μM) to that of the type IIIa. However, they have less affinity for the substrate (table 1), and did not hybridize with the type IIIa probe (Barg *et al* 1990).

The N-terminal sequence of the IIIb has been elucidated and shown not to be directly related to the type IIIa, notwithstanding the similarities in biochemical properties (Thomson, Barg and Amyes 1990). The nucleotide sequence of IIIc has also recently been detemined and again shares little homology with the IIIa. Although it shares some homology with the *E. coli* chromosomal DHFR it seems most closely related to the DHFR of *Halobacteria volcannii* (S.G.B. Amyes personal communication).

1.5.5d. The Type IV Dihydrofolate Reductase

During a survey of urinary pathogens from Vellore, India (Young *et al* 1986a), a group of plasmids were identified that conferred unusually low resistance to trimethoprim in Isosensitest agar or minimal agar containing methionine, glycine and adenine, MIC 5mg/L, but showed increased resistance when tested on minimal agar, MIC 160mg\L (Young *et al* 1986b).

It was speculated that the presence of the metabolites were having an effect on the expression of the type IV DHFR (Young *et al* 1986b) by their influence on the metabolic pathway leading from THF to 5,10-methyl THF and the formation of thymine (as described previously). It has been shown that the presence of the DHFR IV in *E. coli* K12 J62-2 allows the cells to grow in the presence of 10mg/L trimethoprim in minimal media, but cell death occurs in this concentration of drug if methionine, glycine and adenine are present (Young *et al* 1993). The DHFR was subsequently shown to be inducible at trimethoprim concentrations of greater than 10mg/L; up to 600-fold increase in production of the enzyme is seen, a unique property of this DHFR (Young and Amyes 1986a). It has been speculated that it is the over-production of the enzyme that confers resistance on the cell since the enzyme is not very resistant to trimethoprim (table 1)(Thomson, Young and Amyes 1993).

The induction mechanism itself also appears to be less than straightforward. Induction of the enzyme does not seem to be a direct response to the presence of the drug but rather a response to the metabolic effect imposed by trimethoprim. Induction of the enzyme does not occur until between 5 and 14 hours after inoculation of broth containing trimethoprim (Thomson *et al* 1993). The induction of the enzyme has also been shown to be dependant on the presence of methionine, glycine and adenine (Thomson *et al* 1993). This would seem to contradict the MIC results. The MIC is lower in media containing methionine, glycine and adenine than in minimal media; it might then be expected that the enzyme will be induced in minimal media but be lower in media containing methionine, glycine and adenine. The presence of thymidine completely abolishes the effect and cell death and morphological changes associated with thymine starvation can be observed in the cells prior to induction of the DHFR. Thymine deficiency, therefore, seems to play a role in mediating the induction of the enzyme (Thomson *et al* 1993).

The enzyme has been shown to be larger than the other DHFRs, M_r 46,000, although this may be an over estimate and the actual M_r may be only 36,000 (Thomson Young and Amyes 1990), and it is only partially resistant to trimethoprim, ID_{50} 0.2μM (Young and Amyes 1986a).

This induction mechanism seems to be quite unlike the chromosomal induction mechanism described by Tennhammar-Ekman *et al* (1986) and Flensberg and Sköld (1987), in which the response to trimethoprim is immediate and thymine is not thought to influence expression.

The type IV DHFR has not been encountered elsewhere but this may be because, as with the case of the type III, low level trimethoprim resistance is not followed up as closely as high level resistance. This may be even more applicable to the type IV given the very low levels of resistance shown on convential media. A DNA probe is available for this enzyme and, although this includes large regions outside the structural gene, little cross-hybridisation is seen with the other standard DHFR-carrying plasmids (Towner *et al* 1988).

1.5.5e. The Type V Dihydrofolate Reductase

The DHFR type V was originally isolated in Sri Lanka where it was shown to be the most common enzyme mediating plasmid-borne trimethoprim resistance (Sundström *et al* 1987). It has since been isolated sporadically in Europe and the US. Less than 0.3% of trimethoprim resistant enterobacteria were reported to harbour the type V in a study conducted in Finland (Heikkilä 1990a), while 4% of enterobacteria from a children's day care centre in the US carried this enzyme and Towner *et al* (1991) reported only slightly higher levels in Nottingham at 9.9% in the period 1978-83 and 5.8% in 1987-88. However, a study in Sicily reported that all *Shigella sonnei* from an outbreak of trimethoprim resistant shigellosis harboured only the DHFR type V. This was mediated by the spread of only two plasmids and these plasmids or the original infection may have originated in Sri Lanka (Agodi *et al* 1990), indicating a localized spread of the enzyme. An investigation of trimethoprim resistant *Shigella* spp. in Finland reported that in only 5 of 175 strains was the DHFR type V implicated; in four of these cases the patients had recently returned from Sri Lanka (Heikkilä *et al* 1990a).

The enzyme itself has biochemical properties very similar to the type I DHFR (Towner *et al* 1990) (table 1). When sequenced it was shown to be closely related to the type I, having 84% similarity and sharing 75% identity (Sundström *et al* 1988; Singh *et al* 1992).

The DNA probe for this gene is 500bp long and is comprised of almost only the structural gene, providing a very specific probe which under conditions of high stringency shows no hybridisation to the other DHFRs, even the closely related type I (Towner *et al* 1988).

The *dhfr*V gene is found in the transposon Tn*21* and as such is associated with sulphonamide resistance, an enormous genetic advantage if the trimethoprim-sulphonamide combination is in use. As part of the Tn*21*-like transposon group the *dhfr*V has been identified as a "gene-cassette" in an integron structure (Sundström *et al* 1988). Although the distribution of the DHFR type V has been wide its prevalence remains low in most parts of the world.

1.5.5f. The Type VI Dihydrofolate Reductase

There has been only one reported report of this gene, in a strain of *Proteus mirabilis* from South Africa (Wylie *et al* 1988). It is similar to the type I in sequence, sharing 63% homology with the type I and the type V, (Wylie and Koornhof 1991). It does not, however, cross-hybridise with the other DHFR gene probes (Towner 1990). It is also more resistant to trimethoprim, reflected in a higher Tp ID_{50} and K_i (table 1) (Wylie *et al* 1988). The DNA probe for this gene has not been available so nothing is known of its epidemiology.

1.5.5g. The Type VII Dihydrofolate Reductase

Originally identified in a porcine isolate of *E. coli*, the type VII DHFR is similar to the type Ia, Ib and V DHFRs in its biochemical properties (Amyes *et al* 1989). An intragenic DNA probe has been produced which is 300bp in length and which shows some degree of cross-hybridisation with the types I and V indicating close similarity of the enzymes at the DNA level (Towner and Carter 1990). The nucleotide sequence of the *dhfr*

VII gene, located on transposon Tn5086, has recently been determined and has been shown to be related to the types I, V and VI, having between 64 and 71% homology with these *dhfr* genes (Sundström *et al* 1993).

Although originally identified in an animal isolate, there is evidence of a shared pool of R-factors in animal and human bacteria (Towner *et al* 1986) and the type VII has subsequently been identified at low frequency in human isolates from Finland (Heikkilä *et al* 1990a) and seems to be increasing in prevalence in Nottingham, where it was first identified, from 1.2% of trimethoprim resistant *E. coli* in 1978-83 to 7.2% in 1987-88 (Towner *et al* 1991). The enzyme was also isolated in Scandinavia where it has been sequenced and shown, as predicted by biochemical and hybridisation data, to be closely related to the types I and V (Sundström *et al* 1993). This gene, like the *dhfr*I and *dhfr*V, is associated with a transposon. The *dhfr*VII has been shown to be part of the Tn*21*-like transposon Tn*5086*, which like Tn*21* has an associated integron which may have been responsible for the location of *dhfr*VII in the transposon. The transposon also mediates resistance to quaternary ammonium compounds, mercuric ions and sulphamethoxazole. The *dhfr*VII, and the transposon have been shown on a plasmid, R22259, isolated originally in Sweden in 1974 (Sundström *et al* 1993); thus despite its recent characterization the gene has been in existance for almost 20 years.

1.5.5h. The Type VIII Dihydrofolate Reductase

The type VIII mediating high level resistance has been reported only once. It has a sequence that marks it as being different from the type I-like enzymes, the V, VI and VII (Sundström 1989)

1.5.5i. The Type IX Dihydrofolate Reductase

Like the type VII, this DHFR was originally isolated in a porcine strain. It was initially identified in two distinct plasmid types in Sweden (Jansson and Sköld 1991). This enzyme mediates only moderate levels of resistance to trimethoprim, MIC 250mg/L, even though it has an ID_{50} of 20μM compared to 57μM for the type I; it was speculated that the lower resistance was as a result of lower expression of the gene (Jansson and Sköld 1991). The type IX shares some

homology with the type I (29%), the type III (31%), and the *E. coli* chromosomal enzyme, and seems to represent an intermediary resistance mechanism (Jansson and Sköld 1991). It has, so far, been isolated in only one human strain but was reported in 11% of porcine trimethoprim resistant *E. coli*, in Sweden; approximately 60% of resistance was the result of the presence of the type I enzyme (Jansson, Franklin and Sköld 1992). Its wider spread into human bacteria may be limited because of the success of the type I DHFR.

1.5.5j. The Type X Dihydrofolate Reductase

The type X DHFR has recently been isolated in Australia. It has been sequenced and appears to be located on an unusual integron, In7, and as such is associated with sulphonamide resistance. It shows homology with other DHFRs, up to 28% identity, 45% similarity. The putative enzyme has a molecular weight of 21,000 and from the sequence data appears not to directly related to any other DHFRs, although it may have a similar molecular weight. The spread of the gene is being monitored and a probe derived from the sequence will be available (Parsons *et al* 1991).

1.5.5k. The Type XII Dihydrofolate Reductase

The last of the recently isolated *dhfr* genes in gram-negative rods, isolated from an *E. coli* strain in the normal flora of children from a child day care centre in Texas, has also only been characterized by its sequence (Singh *et al* 1992). Like the type X nothing is known of the biochemical properties of the enzyme. The gene shares some homology with the other *dhfr*s, 60% similar, 35% identical to the type III; 59% similar, 39% identical to the type V; 56% similar, 32% identical to the type I; and 57% similar, 36% identical to the *E. coli* chromosomal enzyme; it would again seem not to be directly related to any of the other *dhfr*s (Singh *et al* 1992). It confers resistance at an MIC of 500mg/L and in the study was reported to be fairly widespread in the sub-population under investigation, being present in 21% of the trimethoprim resistant *E. coli* (Singh *et al* 1992).

1.5.5l. The Type SI Dihydrofolate Reductase in *Staphylococcus*

The only DHFR resistant to trimethoprim so far identified in Staphylococci is the type S1 (Amyes and Tait 1990). First identified and characterized (Young *et al* 1987) in an Australian Multi-resistant *S. aureus* (MRSA), the enzyme confers high level resistance, being produced in high quantities and is resistant to trimethoprim, ID_{50} 50μM. It has a molecular weight equivalent to that of the staphylococcal chromosomal enzyme (Young *et al* 1987). A plasmid mediated DHFR from Staphylococci was also isolated in the US (Coughter *et al* 1987), and this has also been demonstrated to be a type S1 (Tait and Amyes 1989). The S1 has also been demonstrated in strains from Eastern Europe (Hartmann *et al* 1988), London (Amyes and Tait 1990) and in other species of *Staphylococci* (Tait and Amyes 1989).

The DNA sequence has shown the gene to be related to the chromosomal enzyme (Hartmann *et al* 1988; Rouch *et al* 1989) and is associated with insertion sequences responsible for transposition into the chromosome (Rouch *et al* 1989) where it is expressed at a reduced level (Amyes and Tait 1990).

TRANSPOSONS AND INTEGRONS

1.6.1. Transposons

The first report by Fleming in 1972 that trimethoprim resistance was plasmid mediated has been followed by many studies that have demonstrated the increasing prevalence and dissemination of trimethoprim resistance (Towner 1982; Kraft *et al* 1986; Amyes 1989). However, four years after the initial demonstration of plasmid mediated trimethoprim resistance, it was shown that the *dhfr* I gene, of R483, was located on a transposon, Tn*7* (Barth *et al* 1976). Transposition of antibiotic resistance genes was first demonstrated in ampicillin resistance by Hedges and Jacob (1974). Since then transposons have been shown to be widespread and responsible for numerous resistance mechanisms (Grinsted 1986), and are perhaps responsible for the acquisition of resistance by clinical plasmids (Datta and Hughes 1983).

Two classes of transposon have been shown to mediate resistance in gram-negative rods; class I, or composite, and class II, or complex transposons, that differ in both structure and function (Galas and Chandler 1981; Harshey and Bukhari 1981; Schmitt 1986). The class I transposons are a heterogeneous group of elements that carry a variety of resistance mechanisms but share little homology at the DNA level (Grinsted 1986). Their structure is relatively simple. They are formed from two insertion sequences (IS), the basic transposable element, that occur in either direct or indirect repeats between which is situated the resistance gene. IS elements are the regions that code for the *transposase*, the enzyme responsible for recombination (Schmitt 1986). These elements are flanked by inverted or direct repeat sequences of between 8 and 40 base pairs (bp) and provide recognition sequences for the transposase, thus four repeat sequences are available as recombination sites so a number of conformational outcomes are possible (Grindley and Reed 1985).

A conservative mechanism of action has been proposed for the transposition of class I elements (Morisato and Kleckner 1984; Schmitt 1986) in which the transposon is excised from the donor replicon at the repeat sequences and integrated at the target site, destroying the donor in the process. However, a process has also been proposed in which donor and target replicons form a co-

integrate; replication of this structure then takes place before resolution of the replicated transposons, leaving a complete transposon in both replicons (Shapiro 1979). However, this seems a more likely mode of action of the class II or complex transposons (Schmitt 1986). The entire class II element is flanked by inverted repeat sequences of between 30 and 40 bp, that again serve as recognition sequences for the transposase. The enzymes responsible for transposition are not found flanking the resistance genes but are found to one end. The transposition enzymes are the products of genes *tnp*A, the transposase, and *tnp*R, the resolvase responsible for the resolution of the replicated co-integrate of donor and target DNA formed by the transposase at a site *res* (Kleckner 1981); a third gene *tnp*M is involved in regulation of transposition (Schmitt 1986).

1.6.2. Integrons

Recently it has been recognized that a number of antibacterial resistance mechanisms are present in the same, or closely related class II transposons, forming a group of transposons like Tn*21*(Wiedemann *et al* 1986; Martinez and de la Cruz 1988). These transposons, together with the Tn*3* family, are the major contributors to multi-resistance plasmids (Grinsted *et al* 1990). Resistance to sulphamethoxazole is usually present; OXA and PSE β-lactamases are frequently present (Lafond *et al* 1989) as are a variety of transferase genes mediating resistance to aminoglycosides (Wohileben *et al* 1989; Hall and Vockler 1987). Resistance to mercuric compounds is also a common feature of these structures (Brown *et al* 1986). The trimethoprim resistance genes *dhfr* IIc (Fling and Elwell 1980) and *dhfr* V (Sundström *et al* 1988) were both identified in Tn*21*-like transposons.

It is not immediately obvious how so many different resistance mechanisms can be located in the same transposon. Tn*21* and similar elements are capable of acquiring novel resistance mechanisms and resistance to new drugs, indicating a greater level of sophistication (Zuhlsdorf and Weidemann 1992). It has been recognized that these structures have been formed by the insertion of resistance genes into already existing transposons by a *rec*A independent recombination system known as *integration* (Martinez and de la Cruz 1988).

In the transposons, and plasmids carrying similar structures without the presence of the transposon, e.g. R388 and R46 (Sundström *et al* 1988), highly conserved regions are found in sequences immediately surrounding the structural gene. Flanking the structural gene for antimicrobial resistance are GTTRRRY sequences which mark the insertion points for the resistance gene and represent recombinational "hot-spots" for cross-over of genes, forming "gene-cassette" structures (Schmidt *et al* 1989; Martinez and de la Cruz 1990). At the 5'end of the resistance gene there is a conserved region that contains a common promoter immediately upstream of the insertion site, thus any inserted genes are transcribed from this common promoter (Stokes and Hall 1989), and 5' to this is an open reading frame (ORF) which codes for a protein which shares homology with both transposon resolvases and phage site-specific integrases (Oullette and Roy 1987; Mahillon and Lereclus 1988). This *integrase* has been designated *int* (Stokes and Hall 1989) or *tnp*I (Mercier *et al* 1990).

At the 3'end is the active site for the insertion of new genes by the integrase (Martinez and de la Cruz 1990) and in the correct orientation for transcription from the common promoter (Collis and Hall 1992). This is an imperfect palindromic region of about 59 bp, the structure of which has been shown to be quite variable in length and sequence (Zuhlsdorf and Weidemann 1992), which has at its 3'end the "hot-spot" GTTRRRY sequence, into which the new gene will insert by cross-over at the GTTRRRY sequences of the in-coming "gene-cassette" and the target sequence (Collis and Hall 1992).

These elements have been called *integrons* (Stokes and Hall 1989) and appear to be responsible for the accumulation of many resistance genes in plasmids and Tn21-like transposons (Bissonnette and Roy 1992). Evidence of an ancestral integron has been found in plasmid pSV1, a plasmid of *P. aeruginosa*. This consists of only the integrase region and a single GTTRRRY cross-over point (Bissonnette and Roy 1992). This "primitive" integron lacks the 59bp region, which may indicate that this region was initially associated with an in-coming resistance gene (Bissonnette and Roy 1992), since this is necessary for the integration of new genes (Martinez and de la Cruz 1990). This will only have been necessary once, future genes being integrated into this site by the action of the integrase, which can act in *trans* (Grinsted *et al* 1992).

There is evidence that the integration of the *sul* gene was an early event in the formation of antimicrobial resistance integrons, this being highly conserved in these structures, probably as a response to the early use of sulphonamides (Bissonnette and Roy 1992). This has been followed by the evolution of these structures, not only by the action of integrases but by homologous recombination and location in transposons (Bissonnette and Roy 1992), leading to the diversity seen in the Tn21 group of transposons (Zuhlsdorf and Weidemann 1992).

1.6.3. Trimethoprim Resistance Genes Associated with Transposons and Integrons

Trimethoprim resistant *dhfr* genes have been found associated with transposons and integrons, leading to a variety of genetic backgrounds for the trimethoprim resistant DHFR enzymes (Sundström *et al* 1988; Sundström and Sköld 1990; Heikkilä *et al* 1991). The *dhfr*V was initially described in association with Tn*21* and was observed to be flanked by regions similar to those flanking the *dhfr*II of R388 (Sundström *et al* 1988). These regions have been shown to be associated with integron function, the *dhfr*V forms a "gene-cassette" (Sundström *et al* 1988). Thus, these DHFRs have been linked to sulphonamide resistance, an advantageous event if the trimethoprim-sulphonamide combination is present. The *dhfr*I has also been identified located in a Tn21-like transposon, again linked with sulphonamide resistance (Sundström *et al* 1988). The *dhfr*I also forms a "gene-cassette" structure, flanked by GTTA sequences like the GTTRRRY sequences previously reported, as part of Tn*7* which also seems to possess integron related sequences, a 59bp region and an integrase ORF (Sundström and Sköld 1990). The integration of other resistance genes in Tn*7* has been observed (Tietze *et al* 1987) and spectinomycin resistance in Tn*7* is also associated with a gene-cassette, so Tn*7* may have, although it has not been directly observed, an integron function which is similar to that of Tn*21* (Sundström and Sköld 1990; Sundström *et al* 1991).

The *dhfr*VII, which is very similar in properties and sequence to the types I and V (Thomson 1990; Towner and Carter 1990; Sundström *et al* 1993) is also associated with a Tn*21*-like integron system (Sundström 1993; Bissonnette and Roy 1992). The *dhfr*VII is located on a Tn*21*-like transposon Tn*5086*, which also encodes resistance genes to quaternary ammonium compounds, mercuric ions and sulphamethoxazole. The *dhfr*VII is found as a gene-cassette, like the *dhfr*I and

*dhfr*V, which seems to have replaced the *aad*A1 gene-cassette for spectinomycin/streptomycin resistance. Tn*5086* also differs from Tn*21* in having a deletion of approximately 4.2Kb upstream of the *dhfr* gene, a region in which deletions and rearrangements are frequently seen (Sundström *et al* 1993). The *dhfr*X has been characterized as part of an integron, In*7*, which also has the sulphonamide resistance gene (Parsons *et al* 1991). Transposon and integron mediation of trimethoprim resistance has contributed to the evolution and dissemination of the mechanisms of resistance, and the evolution of these mobile elements, particularly integrons, may be the result of antibiotic pressure.

INDUCIBLE RESISTANCE MECHANISMS IN GRAM-NEGATIVE RODS

1.7.1. The Inducible Chromosomal β-lactamases

Pseudomonas aeruginosa and most enterobacteria have chromosomal β-lactamases. In *Enterobacter* spp., *Citrobacter* spp., *Providencia* spp., *Morganella*, indole positive *Proteus* spp. and *P. aeruginosa* these enzymes are inducible (Sykes and Matthews 1976). The chromosomal β-lactamases of the enterobacteria share considerable homology but have quite different substrate profiles (Sykes and Smith 1979). The various β-lactams differ in their ability to induce the enzymes and in their lability to hydrolysis by them (Livermore 1987). Ampicillin, the first generation cephalosporins and benzylpenicillins are strong inducers at concentrations well below the MIC (Livermore 1987). When induction occurs high levels of resistance are seen, *in vivo* and *in vitro*. High MICs are produced in both broth and solid media. The increase in enzyme synthesis occurs, in exponentially growing cells, immediately after addition at sub-inhibitory levels of the drug; high levels of enzyme expression are seen after four hours (Minami *et al* 1980).

The induction mechanism has not been fully elucidated; however, a number of genes seem to be involved in the control of *amp*C, the β-lactamase structural gene (Korfmann and Sanders 1989). Adjacent to the *amp*C gene is *amp*R, these genes have promoters which are divergent and over-lapping. The *amp*R gene product binds to this region in an inactive form in the absence of inducer, regulating itself and *amp*C (Lindquist *et al* 1989). In the presence of inducer, the *amp*R product is activated by a ligand, that seems to be provided by a third gene product, *amp*G (Korfmann and Sanders 1989), which increases the transcription of *amp*C (Lindquist *et al* 1989). In the absence of inducer, this ligand, so far unidentified, is inactivated by the *amp*D gene product (Korfmann and Sanders 1989). Thus *amp*C is negatively controlled by the action of *amp*D (Korfmann and Wiedemann 1988). The expression of *amp*D is somehow controlled by the inducer; it seems unlikely that this is by direct action of the β-lactams since there is no evidence that these agents can penetrate into the cytoplasm. Another protein, probably a trans-membrane protein, must be involved in transmitting the signal to increase *amp*D expression. There is some evidence that PBP2 is involved in the regulation of *amp*D (Oliva *et al* 1989).

1.7.2. The Induction of Tetracycline Resistance

Tetracycline resistance in the enterobacteria is mediated by a inducible mechanism situated on transposons Tn*10* and Tn*1712*, which have been shown to be related (Chopra 1986). The resistance determinant in these transposable elements consists of two regions, a membrane bound protein of M_r 36,000 which mediates active efflux of the drug, and a repressor protein. There are two operators that control the over-lapping promoters for the repressor and the membrane protein (Chopra 1986). The repressor sensitive operators thus control transcription of the resistance determinant and the repressor (which is thus autogenously controlled). The repressor will act *in trans* to the operator region (Klock *et al* 1985) and is sensitive to the presence of tetracycline. Thus tetracycline directly effects the expression of resistance.

Phenotypically high level resistance is seen *in vivo* and *in vitro*, MICs are high in broth and agar dilution methods. This system probably evolved in the wild to deal with tetracycline produced by streptomyces, having evolved separately in both Gram-negative and Gram-positive organisms (Chopra 1986).

AIMS OF THIS THESIS

Young *et al* (1986a) found very high levels of resistance to trimethoprim and ampicillin amongst urinary tract pathogens isolated in Vellore, south India. A survey was conducted in Vellore, in conjunction with the Christian Medical College and Hospital; to establish the rates of carriage of resistant faecal flora, for the commonly used antimicrobials, trimethoprim, ampicillin, chloramphenicol and nalidixic acid in the population of the town of Vellore and rural villages surrounding the town. It could then be established whether the normal faecal flora could be acting as a reservoir of resistant bacteria and resistance genes.

Those strains that were resistant to trimethoprim will be investigated further. The mechanisms responsible for trimethoprim resistance will be established, and the presence of these mechanisms on plasmids, transposons or integrons will be investigated. Any novel resistance mechanisms to trimethoprim will be identified and characterized. The DHFR type IV has been shown to be peculiar to this region of India; it has not been identified anywhere else. The continued presence of this unusual trimethoprim resistance mechanism will be established and the nature of the mechanism of action investigated further.

CHAPTER 2

MATERIALS AND METHODS

2.1. REAGENTS

Chemicals and reagents were all supplied by Sigma chemicals (Poole, Dorset) unless other wise indicated.

2.2. BACTERIAL STRAINS

The standard laboratory strains used were as follows:

Table 2. Bacterial strains.

Strain	Requirements	Sited in:	From:
E. coli K12	prototroph	Bachmann 1972	S.G.B. Amyes
E. coli K12 J62-2	*pro, his, trp*	Bachmann 1972	S.G.B. Amyes
E. coli DH5α	*thi*	Hanahan 1983	H-K. Young
E. coli JM101	*thi, pro* F'[*pro+*]	Messing 1979	J. Moss
S. typhimurium LT2			S.G.B. Amyes

2.3. MEDIA

2.3.1. Minimal Media

Minimal salts medium was prepared as double strength solution as described by Davis and Mingioli (DM) (1950) (table 3). To prepare diluent for the dilution of cell suspensions, an equal volume of distilled water was added. For the preparation of minimal salts growth media, any auxotrophic requirements were added, aseptically, at the concentrations given in table 4, along with any selective agent and D-glucose to a final concentration of 0.28%. To make a liquid medium the volume was made up with sterile distilled water; for solid media the salts solution was added to sterile molten agar immediately before the plates were poured.

Table 3. Double strength Davis and Mingioli minimal salts.

SALT	For 1L (g)
K_2HPO_4	14.0
KH_2PO_4	6.0
tri-sodium citrate	1.0
$MgSO_4.7H_2O$	0.2
$(NH_4)_2SO_4$	2.0

The salts were dissolved in the order given and made up to 1L in distilled water. Then sterilised by autoclaving at 121°C for 15 minutes.

Table 4. Supplements and requirements.

Auxotrophic requirement	Concentration of stock solution	Final concentration	Sterilization by
L-Histidine	5g\L	50mg/L	steam
L-proline	5g/L	50mg/L	steam
L-tryptophan	2g/L	50mg/L	steam
L-methionine	5g/L	50mg/L	steam
glycine	5g/L	50mg/L	steam
thymidine	5g/L	*Varied	filtration
adenine	5g/L	50mg/L	filtration

Steaming was carried out for 30 minutes. Filtration was through a Millipore filter with a 0.22μm pore size.

*The concentration of thymidine used for each experiment is given in the Results.

2.3.2. Complex Media

The following complex media were used: Isosensitest Broth (CM473), Nutrient Broth No. 2 (CM67), Isosensitest Agar (CM471) and MaConkey Agar (CM7b), all Oxoid (Basingstoke, Hants). Modified Mueller-Hinton agar, for the selection of trimethoprim resistant, lactose fermenting organisms was made up as follows: Mueller-Hinton agar (Difco, East Molesey, Surrey) containing 1% lactose, 0.5% bile salts, neutral red 75mg/L. LB agar and LB broth were supplied by Gibco-BRL (Paisley, Scotland). All media were made to the manufacturers instructions and sterilized at 121°C for 15 minutes.

SOB medium was made up as follows, for one litre:

20g Bacto tryptone (Difco): 5g Bacto yeast extract (Difco): 0.5g NaCl: 10ml of 0.25M KCl. The pH was adjusted to 7.0 with NaOH. SOC was made up as SOB but 20mM glucose was added.

2.4. ANTIMICROBIAL AGENTS

Antimicrobial agents were either supplied as sterile powders from the manufacturer or were sterilized by filtration through a 0.22μm pore membrane (See table 5). In susceptibility testing, and other experiments, involving the amoxycillin-clavulanic acid combination a 2:1 ratio of amoxycillin : clavulanic ratio was used.

Table 5. Antimicrobial agents.

Antimicrobial	Abreviation used here	Supplier
Trimethoprim	Tp	The Wellcome Foundation
Ampicillin	Ap	SmithKline Beecham
Spectinomycin	Sp	Sigma Chemicals
Streptomycin	Sm	Sigma Chemicals
Tetracycline	Tc	Lederle Laboratories
Chloramphenicol	Cm	Boehringer Mannheim
Gentamycin	Gm	Sigma Chemicals
Amoxycillin }	Ag	Bencard
Clavulanic acid }		SmithKline Beecham
Ciprofloxacin		Sigma Chemicals
Nalidixic acid	Na	Sigma Chemicals
Sulphamethoxazole	Su	Sigma Chemicals
Kanamycin	Km	Sigma Chemicals
Cephaloridine	Cp	Glaxo laboratories
Cefuroxime		Glaxo Laboratories
Ceftazidime		Glaxo Laboratories
Cefotaxime		Roussel Laboratories
Rifampicin		Ciba laboratories

2.5. SURVEY OF ANTIBIOTIC RESISTANCE IN THE NORMAL GUT FLORA OF VOLUNTEERS

Volunteers from the urban population of Vellore, a town of some 60,000 inhabitants about 120 km from Madras in Tamil Nadu, south India, were identified and recruited by the community health unit of the Christian Medical College and Hospital (CMCH), in Vellore. Volunteers from three rural villages, about 40 km from Vellore, were identified by the Rural Unit for Health and Social Administration (RUHSA), a unit of the hospital which operates in the rural communities around Vellore. Three villages were used: Kilvayattarankuppam, a roadside village with good communication with other areas and the town; Kavanur a riverside village with less contact with outside areas; and Melmoil, situated in the nearby foothills, which was fairly remote.

The volunteers were each issued with a sterile bottle and asked to provide a faecal specimen for collection the next day. They were also asked a few relevant questions concerning their diet, health, sanitary arrangements and recent medication. Those who had received antimicrobial chemotherapy in the two weeks prior to taking the sample were eliminated from the study. After collection the samples were taken to the laboratory for processing.

The samples were plated out on Oxoid MaConkey agar containing ampicillin, chloramphenicol or nalidixic acid, 10mg/L, and on the modified Mueller-Hinton agar, described above, containing of trimethoprim, 10mg/L. The plates were incubated overnight at 37°C after which resistant lactose fermenting gram-negative rods were selected, colony types and the specimens which had contained them were noted. The strains were then purified on the same media prior to storage in nutrient agar slopes.

2.6. STORAGE OF CULTURES

All strains were stored at -70°C, in cryovials (Alpha laboratories, Eastleigh, Hants). The bacteria were grown overnight, after which an aliquot was added to a vial and mixed with glycerol, to a final concentration of 5%, before freezing. Strains were always sub-cultured from the stock and not passaged.

2.7. ANTIBACTERIAL SUSCEPTIBILITY TESTING

Minimal inhibitory concentrations (MIC), and break-point susceptibility testing were performed on ISTA agar, or on minimal media where indicated. MICs were determined using agar double dilution of the antibacterial. The agar was inoculated with a 2μL spot, delivered from a multipoint inoculator (Denley, Billinghurst, Surrey), of a 1 in 10^4 serial dilution of a fresh overnight culture in nutrient broth. The same procedure was followed for break-point testing but only one drug concentration was used. The concentrations used in break-point testing were as stated in the Results.

2.8. VIABLE COUNTS

Serial, 1 in 10, dilutions were made of the test suspension or culture; 0.1ml aliquots of the dilutions were then spread onto IST agar plates and incubated overnight at 37°C.

2.9. TRANSCONJUGATIONAL TRANSFER OF PLASMIDS

From fresh overnight cultures in nutrient broth, 0.1ml of the donor strain and 1.0ml of the recipient strain were added and gently mixed in 4.5ml of warmed nutrient broth. The mixture was incubated at 37°C for six hours; the cells were then spun down in a Heraeus Christ labofuge and washed in single strength D M. Serial 10-fold dilutions were prepared before spreading 0.1ml of the dilutions on minimal agar containing the appropriate auxotrophic requirements of the recipient and selective drugs. The plates were incubated for 48 hours; any transconjugants were verified by checking auxotrophic requirements and resistance markers.

To select *E. coli* K12 J62-2 trimethoprim resistant transconjugants, media containing the requirements of this strain, proline, histidine and tryptophan, along with rifampicin, 25mg/L, and trimethoprim, 10mg/L, was used. In transfers, from *E. coli* K12 J62-2 to *E. coli* K12 and *S. typhimurium* LT2, the transfer frequency was calculated thus:

$$\text{Transfer frequency} = \frac{\text{No. transconjugants/ml of final mix}}{\text{No. donors in original culture/ml}}$$

2.10. ENZYME PREPARATION AND ASSAY

2.10.1. Preparation for Dihydrofolate Reductase Assays

Crude cell lysates were prepared from overnight cultures in Isosensitest broth, either 200 ml for induction studies, or 1L to prepare cell lysate enzyme for further purification and gel filtration chromatography. The cells were harvested by centrifugation at 6,000r.p.m. for 15 minutes (Sorval RC-5B, DuPont Stevenage, Herts.); the cells were then washed in cold buffer A (50mM sodium phosphate buffer pH 7.4, 10mM β-mercaptoethanol, 1mM EDTA). The cells were finally resuspended in cold buffer A; after this stage all procedures were performed on ice or at 4°C. The cells were disrupted by sonication; two 30 second pulses separated by a 30 second cooling period at 8μm (MSE Soniprep).

This crude enzyme preparation could then be used to assay the enzyme's specific activity, being the amount of DHFR activity as a proportion of the total protein present, thus giving an expression of the DHFR produced by the cells.

To examine the biochemical properties of the enzyme, further purification was necessary. Nucleic acids were removed by precipitation with the addition of 0.1 volume of 10% streptomycin sulphate, followed by centrifugation at 12,000g for 30 minutes. Dihydrofolate reductase was precipitated from the lysate by the addition of ammonium sulphate to 50% and 80%, with the recovery of each precipitate by centrifugation at 12,000r.p.m. for 30 minutes. The resulting 50% to 80% precipitate was then resuspended in a minimum volume of buffer A (about 2ml). The activity was assayed and the preps could then be frozen at -20°C until required.

2.10.2. Preparation of Cell Lysates for β-lactamase Assay

These preparations were carried out in the same way as above, but 50mM sodium phosphate buffer pH 7.0 was substituted for buffer A. To prepare lysates for both assays from the same culture, after the initial harvest, the cells were resuspended in Davis and Mingioli medium; this suspension was split into two. The cells were harvested and the two extracts were then treated as appropriate for the enzyme assay to be performed.

2.10.3. Dihydrofolate Reductase Assays

This was performed as described by Osborn and Huennekens (1958). A Perkin Elmer lambda 2 spectrophotometer, with heated cuvette carriage at 37°C, was used to measure decrease in absorbance at 340nm. This is caused by the oxidation of NADPH to NADP and the reduction of dihydrofolate (DHF) to tetrahydrofolate, by the action of the DHFR. 52% of this combined fall in absorbance is a result of the reduction of DHF, when both substrates are acted upon stoichiometrically (Matthews *et al* 1963). With this qualification, the rate of enzyme activity is expressed in molar terms of DHF reduced to THF.

The reaction was performed in 1ml quartz cuvettes containing:

40mM sodium phosphate buffer pH 6.0
10mM β-mercaptoethanol
0.08mM NADPH
enzyme
distilled water to a volume of 0.95ml

A blank was also prepared but contained no NADPH. Both this and the test were placed in the spectrophotometer and allowed to equilibrate for four minutes. Dihydrofolate was then added to both cuvettes; the absorbance was read for 10 minutes or until it had reached zero. If a decrease in absorbance was recorded before the addition of DHF, this was taken as DHF-independent NADPH oxidase activity. The final rate of DHFR activity was taken as the total rate of change in absorbance less that of any DHF-independent oxidase activity.

2.10.4. Measurement of Activity of β-lactamase

This was measured as a change in O.D. 384nm of nitrocephin (Glaxo laboratories, Middlesex) at a concentration of 100μM and O.D. 238nm of ampicillin (Sigma Chemicals) at a concentration of 10mM, in sodium phosphate buffer at a final volume of three ml. Crude extract was added to the test cuvette and the decrease in absorbance over time was taken with reference to a blank containing substrate but no enzyme.

The activity of the sample was then determined by:

$$R = \frac{\Delta OD \times n \times \text{extract dilution}}{\Delta OD_1 \times \text{time}}$$

R= μM of substrate hydrolysed $minute^{-1}$ ml^{-1} extract.
ΔOD= change in optical density.
n= μM substrate in the cuvette.
OD_1= optical density of the intact substrate.

2.10.5. Protein Estimations

Estimations of protein concentrations in crude lysates were determined by the method of Waddell (1956). The samples to be tested were initially diluted in distilled water. The absorbance was then measured at 215 and 225nm. The concentration of protein could then be ascertained with reference to a previously prepared standard curve. Specific activity was determined from the activity of the sample divided by the total protein concentration.

2.10.6. Purification of Dihydrofolate Reductase Samples by Gel-Filtration

Fine grade Sephadex G75 (Pharmacia, Uppsala Sweden) was swollen in buffer A by maintaining at 100°C for three hours. After allowing the gel to settle, excess buffer was decanted and fresh buffer added. The slurry was poured in an Amicon

acrylic column ($2cm^2$x90cm) which was maintained at 4^0C in an LKB mini cold lab. When fully packed, a peristaltic pump was attached (Pharmacia) and buffer A was washed through the column at a flow rate of approximately 2ml in ten minutes; this was continued for about 48 hours.

Samples were applied to the column and eluted with buffer A at 2ml in 10 minutes, until at least one column volume had been passed. Fractions were collected in an LKB Ultrorac fraction collector. After elution the column was washed for 12 hours before further use.

2.10.7. Determination of Protein Molecular Weight

The sephadex column was calibrated essentially by the method of Andrews (1964). Three proteins of known molecular weight were applied to the column in 1ml aliquots of 5mg/ml. These were eluted at approximately 2ml in 10 minutes, 2ml fractions were collected and the elution volumes of the proteins were determined by measuring the absorbance of the fractions. A standard curve was then plotted of elution volume against molecular weight with which to compare the peak activity of the test elution volume.

2.11. ENZYME KINETICS

After determining the peak activity of the eluted fractions from the column, those fractions containing the most activity were pooled.

Inhibition of enzyme activity by trimethoprim and methotrexate was determined in DHFR assays similar to those described above except that they were conducted in the presence of increasing concentrations of drug.

Temperature sensitivity was determined by pre-treating the enzyme at 42°C for increasing time spans up to 12 minutes. The activity was then assayed.

To establish the enzyme's Michaelis constant (K_m) for dihydrofolate, a measure of the affinity of the enzyme for the substrate, the activity was determined in conditions of partial saturation with DHF. The results were then analyzed by the method of Lineweaver and Burk (1934). The reciprocal of the substrate

concentration, $1/[s]$, was plotted against the rate of enzymic reaction, $1/v$. When extrapolated, the interception on the abscissa gives the negative reciprocal of the Michaelis constant, this being the concentration of substrate at which the rate of reaction is half the maximum velocity.

The inhibitor constant (K_i) for the competitive inhibition of an enzyme was determined by repeating the assays for the K_m but in the presence of trimethoprim. The $1/[s]$ and $1/v$ are plotted as before. The competitive inhibition of the reaction by trimethoprim means that the line crosses the ordinate at the same point as the K_m plot, but the interception with the abscissa is nearer to the origin because the reaction is slowed. This point gives the negative reciprocal of the apparent K_m in the presence of inhibitor, the K_p .The K_i is calculated from:

$$K_i = \frac{[i]}{K_p / K_m}$$

2.12. ANALYTICAL ISOELECTRIC FOCUSING OF β-LACTAMASES

Table 6. Composition of analytical IEF gel.

Material and stock solution	Supplier	Volume (ml)	Final concentration
5% TEMED (tetramethyl-ethylenediamine)	Sigma	0.2	0.25mg/l
40% ampholines(pH3.5-10)	Pharmacia	2.0	2% w/v
33% acrylamide/	BDH	9.0	acrylamide 75g/L
0.9% methylene bis-acrylamide			bis-acrylamide 2g/L
distilled water		25.0	
Riboflavin (20mg/l)	Sigma	4.0	2mg/ml

Stock solutions were made up in sterile distilled water.

β-lactamases were focused on horizontal thin layer polyacrylamide gels containing carrier ampholines of pH 3.5-10.0, by the method of Matthews *et al* (1975). The composition of the gels is given in table 6. To assess the quantity of sample to be loaded on the gel, an empirically derived assay was used to ascertain the activity of

the sample. 30μl of sample were added to 100μl of nitrocephin (50mg/L). The time taken for a colour change to occur could be used as an indication of the activity in the sample. The corresponding volume, in μl, to the time taken in seconds, was used to load the gel.

The samples were loaded near the anode and run at one Watt overnight. The gels were stained with nitrocephin (500mg/L) to allow detection of the focused enzyme.

2.13. DNA TECHNIQUES

2.13.1. Mini-Preparations of Plasmid DNA

Small scale plasmid extractions were carried-out by the alkaline lysis method of Takahashi and Nagano (1984) on 4.5ml of cells grown overnight in nutrient broth at 37°C with vigorous shaking. The extracted DNA was subjected to electrophoresis at 60V overnight in 0.6% agarose in 40mM Tris-acetate pH 8.0, 2mM EDTA electrophoresis buffer (Takahashi and Nagano 1984), in a Gibco-BRL (Paisley, Scotland) Horizon 20.25 horizontal gel tank.

The DNA was visualized by staining for one hour in 50μg/L ethidium bromide and viewed on a U.V. transilluminator (U.V. Products, Cambridge).

2.13.2. Large-Scale Plasmid DNA Preparations

To prepare vector plasmids and plasmids carrying probe inserts, large scale DNA preparations were performed by the polyethylene glycol precipitation method of Sambrook *et al* (1989). Concentration and purity of DNA was assessed by reading the optical density at 260nm and 280nm.

One absorbance unit at 260nm = 50μg/ml for double stranded DNA. The ratio between the O.D. 260nm and O.D. 280nm provides an estimate of purity; the ratio 1:1.8 is an indication of pure double stranded DNA.

2.13.3. Restriction Endonuclease Digests

Restriction enzymes were supplied by Gibco-BRL Life Sciences. DNA was digested as recommended by the manufacturer in the "REact" buffer system supplied with the enzyme. Electrophoresis was performed with the buffer and tanks specified above, but 0.8% agarose was used for the analysis of "wild-type" plasmids. For the analysis or separation of cloned or probe inserts in vector plasmid, a Pharmacia GNA-100 mini-tank was used; an appropriate concentration of agarose was used according to the fragments to be separated or extracted.

2.13.4. Sizing of DNA Fragments and Plasmids

The distance migrated by linear DNA during electrophoresis through an agarose gel is inversely proportional to the log of its molecular weight. A standard curve can be drawn from the distance migrated by DNA fragments of known size against the log of their molecular weight; the unknown can be derived from this curve. Lambda phage DNA digested with *Hin*dIII was the standard used. The molecular weight of plasmids was derived from the sum of the fragments produced on restriction endonuclease digestion.

2.13.5. Southern Blotting

Plasmid DNA was blotted onto Hybond-C Extra membrane (Amersham plc. Amersham, UK), nitrocellulose combined with nylon, by the capillary blotting method recommended by the manufacturer. After overnight blotting the membrane was baked at 80°C under vacuum for two hours.

2.13.6. Colony Blotting

This was carried-out by the procedure of Sundström *et al* (1987). Fresh overnight cultures were picked onto nitrocellulose membrane which was placed on top of an IST agar plate. The patches were allowed to grow for 5 hours. They were then placed on filter paper saturated with the following solutions, with intervening drying on filter paper; 0.5M NaOH for seven minutes; 0.1M NaOH, 1.5M NaCl for 10 minutes; 1M Tris-HCl (pH 7.5) twice for two minutes each; 0.5M Tris-HCl

(pH 7.5), 1.5M NaCl for four minutes. The filter was then air dried before baking at 80°C for two hours under vacuum.

2.13.7. The Preparation of DNA Probes

DNA probes were prepared from those plasmids given in table 7. The plasmids were digested with the appropriate enzymes and the fragments were separated on gels as outlined previously. The probe DNA fragments were extracted from the gel by the Prepagene DNA purification kit (Biorad, Hemel Hempstead, Herts.). Purity and concentration of the fragment was assessed by electrophoresis and comparison with DNA of known concentration.

The DNA probes were labelled with biotin-14-dATP (Gibco-BRL), or [^{32}P]-dCTP (Amersham) with the Gibco-BRL Nick Translation kit, following the manufacturer's instructions.

Table 7. Probes used, and their origin.

Probe for	Derived from	Fragment used as probe	Described by
*dhfr*I	pFE872	*Hpa*I 499bp	Fling & Richards 1983
*dhfr*II	pFE700	*Eco*RI 800bp	Mayer *et al* 1985
*dhfr*III	pUN972	*Eco*RI, *Pst*I 700bp	(Gifted by K.J. Towner)
*dhfr*IV	pUK1148	*Cla*I 1700bp	Towner *et al* 1988
*dhfr*V	pLKO9	*Hinc*II 500bp	Towner *et al* 1988
*dhfr*VII	pUN1056	*Eco*RV 300bp	Towner and Carter 1990
*tnp*A of Tn*21*	pGS150	*Eco*RI 1250bp	Sundström and Sköld 1990
integrase -like ORF of Tn*21*	pLKO26	*Ava*I, *Hpa*I 1700bp	Sundström and Sköld 1990
integrase -like ORF of Tn*7*	pRSSO11	*Bam*HI, *Kpn*I 1289bp	Sundström and Sköld 1990

The plasmids were purified and fragments separated and labelled as described in the text.

Table 8. Control plasmids

Plasmid	Identified by	Enzyme	Originally characterized in
R6K	Datta and Kontimichalou 1965	TEM-1	Hedges *et al*1974
R483	Hedges *et al* 1972	DHFRIa	Sköld and Widh 1974
R67	Acar *et al* 1977	DHFRIIa	Pattishall *et al* 1977
R388	Datta and Hedges 1972	DHFRIIb	Amyes and Smith 1974b
R751	Jobanputra and Datta 1974	DHFRIIc	Fling and Elwell 1980
pAZ1	Joyner *et al* 1984	DHFRIIIa	Fling *et al* 1982
pUK1123	Young *et al* 1986	DHFRIV	Young and Amyes 1986a
pUN433	Towner and Carter 1990	DHFRV	Thomson *et al* 1990
pUN1043	Towner *et al* 1990	DHFRVII	Amyes *et al* 1989

The above plasmids (table 8) were used, in southern blotting, to confirm the specificity and efficacy of the *dhfr* and other gene probes listed in table 7.

2.13.8. Hybridisation of DNA Probes with Southern Blots

Prior to hybridisation with the probe, the membrane was incubated with pre-hybridisation solution, in Gibco-BRL hybridisation bags, for two hours at 42°C. After the removal of the pre-hybridisation solution, hybridisation solution was added containing the probe which had been de-natured by boiling for 10 minutes. Hybridisation was carried-out overnight at 42°C in the solution given in table 10, with overnight incubation. These are as recommended by Gibco-BRL, the manufacturer of both the labelling and detection kits.

High stringency post-hybridisation washes of the membrane were as follows:
1. 2 x SSC, 0.1% SDS for three minutes at room temperature, repeated twice.
2. 0.2 x SSC, 0.1% SDS for three minutes at room temperature, repeated twice.
3. 0.16 x SSC, 0.1% SDS for 15 minutes at 50°C, repeated twice.

All were done with gentle agitation. The membrane was then washed twice with 2 x SSC. Positive hybridisation signals were then detected by means of the BluGene biotin detection kit (Gibco-BRL), following the procedure recommended by the manufacturer. Stock solutions for hybridization mixes were as given in table 9.

Table 9. Stock solutions.

20 x SSC	100 x Denhardts
3.0M NaCl	2% Ficoll
0.3M Sodium Citrate	2% polyvinylpyrolidone
(Ph 7.0)	2% BSA
	in 3 x SSC

Table 10. Stock solutions, pre-hybridisation and hybridisation solutions.

Stock solutions	Pre-hybridisation	Hybridisation
*100% Formamide	50%	45%
20 x SSC	5x	5x
100 x Denhardts	5x	1x
500mM Sodium phosphate (pH 6.5)	50mM	20mM
10% SDS	0.1%	0.1%
10mg/ml sheared herring sperm DNA	1mg/ml	0.5mg/ml
20% Sodium Dextran Sulphate		5%

All stock and final solutions were made up with de-ionized distilled water.

*Formamide was deionised before use with Amberlite Monobed resin M.B.1 (Merck, Poole, Dorset).

*Stocks stored frozen at -20^{0}C.

2.13.9. Cloning DNA Fragments

The vector and target DNA were digested with an appropriate enzyme and the digests were checked by electrophoresis. The DNA was then extracted with phenol and precipitated with ethanol. The ligation reactions were carried-out in 20μl reaction volume, containing ligation mix (Gibco-BRL), digested vector and target DNA, to which was added 0.1 unit of T_4 DNA ligase (Gibco-BRL). The mix was

incubated at 12°C overnight after which a sample was transformed into the host cell.

To ligate an *Eco*RI linker molecule (Boehringer Mannheim) to an extracted DNA fragment, 0.5μg of the linker was incubated with 3μg of the target fragment in a 10μl final volume with 2 units of ligase and incubated overnight at 4°C. This mixture was then digested with *Eco*RI and purified with the Prepagene kit before ligation into the vector plasmid and transformation of a host.

2.13.10. Transformation

The cells to be transformed were grown overnight in 10ml of SOB broth containing 10mM $MgCl_2$; 0.5ml of this culture was then inoculated into 50ml of SOB broth containing 10mM $MgCl_2$, the cells were then grown at 37°C with vigorous shaking to approximately mid-log phase. The cells were then harvested by centrifugation at 2,000g, for 12 minutes, at 4°C (RC-5B Sorval) and resuspended in 12ml ice-cold 50mM $CaCl_2$; the cells were then stored on ice for 15 minutes. The cells were centrifuged as before then resuspended in 4ml of ice-cold 50mM $CaCl_2$ and dispensed in 0.2ml aliquots. The DNA, 5μl of the ligation mix, was added to the cells and gently mixed before storing on ice for 30 minutes. The cells were subjected to a 42°C heat pulse for one minute, after which they were returned to the ice and 800μl of SOC broth was added. They were then allowed to recover by incubation for one hour at 37°C. The cells were then spread onto IST agar containing the appropriate antibiotics. To test the efficiency of transformation, a known concentration of pUC18 DNA was transformed and the number of transformants on agar containing ampicillin, 10mg/L, counted.

The above procedure was used for the transformation of DH5α. To transform J62-2 the same procedure was followed except that purified plasmid DNA from a previously transformed strain was used. This modification was required because the efficiency of transformation of J62-2 was not sufficient to allow direct transformation with the ligation mix.

For M13 subcloning the same procedure was followed except 200μl of SOC was substituted for the 800μl of SOB. The cells were then added to 3ml of soft LB agar (Gibco-BRL) containing 40μl of X-gal (20mg/ml), 4μl of IPTG (200mg/ml) and

0.2ml of an overnight culture of the host cells (JM101). This was gently mixed before pouring over an LB agar plate (Sambrook *et al* 1989). Clear plaques were identified and used to transfect stock cultures. Possible recombinants were sub-cultured onto selective plates followed by plasmid preparation and analysis by restriction digestion.

2.14. VECTOR PLASMIDS

The plasmids used in cloning were pUC18 (Gibco-BRL), pSU18, chloramphenicol resistant (Bartolomé *et al* 1991) and pSC101 (S.G.B. Amyes) a low copy plasmid which carries chloramphenicol and kanamycin resistance. M13mp19 (Gibco BRL)(Yanisch-Perron *et al* 1985) was also used as a vector prior to sequencing.

2.15. DNA SEQUENCING

Single and double stranded DNA sequencing was carried-out with the Sequenase system (Cambridge Bioscience, Cambridge UK) and [^{35}S]-dATP label (Amersham). The M13 -40 forward primer and the M13 -40 reverse primer were supplied by Cambridge Bioscience.

A specific primer, ACATCATGATTGGTATGAT, which was taken from a region immediately upstream of the *dhfr*IV structural gene, was supplied by Edinburgh University Department of Chemistry, Kings Buildings, Edinburgh. The sequence for this primer was kindly given by Dr H-K Young, Dundee University School of Biological Sciences.

Double stranded DNA, pUK1227, was prepared by the Qiagen midi-plasmid DNA preparation kit (Qiagen, Chatsworth, California). Single stranded viral DNA was prepared by the polyethylene glycol precipitation of Sambrook *et al* (1989).

Hydrolink, "Longer-ranger", polyacrylamide gel (Hoeffer, Newcastle-Under-Lyme) made up to the manufacturers recommendations was used to run the sequencing reactions on a Biorad sequencing apparatus. Autoradiography was carried-out with an Amersham autoradiography cassette and Amersham Hyperfilm MP, with 24 hour exposure at room temperature. The development of the film was kindly performed by the X-ray department at the Edinburgh Royal Infirmary.

CHAPTER 3.

RESULTS FROM THE SURVEY OF RESISTANCE IN NORMAL FAECAL FLORA

3.1. ANTIBIOTIC RESISTANCE IN AEROBIC LACTOSE FERMENTING GRAM-NEGATIVE RODS FROM NORMAL FAECAL SAMPLES

3.1.1. The Results of the Survey of Normal Faecal Samples

Faecal samples provided by populations from the town of Vellore and three nearby villages, Kilvayatarrankuppam, Kavanur and Melmoil, were surveyed, as outlined in Materials and Methods, for the carriage of gram-negative lactose-fermenting rods resistant to trimethoprim, ampicillin, chloramphenicol and nalidixic acid. The rate of carriage of resistant organisms in these populations was then determined before the isolated organisms and the resistance mechanisms carried by them were characterized further.

Very high levels of resistance were recorded in both the rural and urban populations to the commonly used antimicrobials trimethoprim, ampicillin and chloramphenicol. Almost every volunteer in the study carried lactose-fermenting gram-negative rods resistant to these agents. Resistance to nalidixic acid was encountered far less frequently in both rural and urban groups; the rural group showed comparatively few members harbouring nalidixic acid resistant bacteria. The urban population had rather more bacteria resistant to nalidixic acid, but not to the same extent as the other agents.

Table 11. Percentage of survey population harbouring resistant gram-negative lactose-fermenting rods.

% of volunteers with resistant organisms	Sample number	Trimethoprim	Ampicillin	Nalidixic acid	Chloram-phenicol
Rural villages					
KVK	43	97.7	93.0	16.3	95.5
Kavanur	46	95.6	100.0	19.6	95.6
Melmoil	38	97.4	97.4	2.7	97.4
Average		96.9	96.8	12.9	96.0
Urban					
Vellore	95	100	98.9	34.7	98.9

KVK is Kilvayatarrankuppam

There was no difference between the villages, despite the various locations, nor was there a significant difference in the rate of carriage of resistant organisms between the urban and rural populations, except in the case of nalidixic acid. Carriage of organisms resistant to trimethoprim, ampicillin and chloramphenicol was the same in the two sample populations (table 11).

Table 12. Average number of different resistant colony types in each specimen.

	Trimethoprim	Ampicillin	Nalidixic acid	Chloram-phenicol
Rural village				
Kilvayattarrankuppam	2.1	1.6	0.3	1.6
Kavanur	1.8	1.8	0.2	1.7
Melmoil	1.3	1.9	0.0	1.6
Average	1.7	1.8	0.2	1.6
Urban				
Vellore	1.8	2.1	0.5	1.9

Not only were the levels of carriage of resistant organisms very high but the samples themselves frequently had more than one distinguishable resistant strain identifiable by differences in colony type. On average each sample carried more than one type of gram-negative rod resistant to the antimicrobial tested, with the exception of nalidixic acid (table 12).

3.1.2. Possible Contributory Factors to the High Rates of Carriage of Resistant Organisms

General levels of hygiene and sanitary provision in this part of the world are low. Drinking water came from bore-holes or wells in the rural villages and from municipal supplies from taps in the town of Vellore, although wells were also used. In the villages there was no sewage or waste disposal facilities; the villagers used the fields. In the town, open sewers were the rule and were obvious in most streets.

In the questionnaires that were filled-out, many of the volunteers reported illness and various symptoms; these are summarized in table 13. According to the local workers with RUHSA, this incidence of illness is quite usual in both urban and rural populations. Specific symptoms are listed; the miscellaneous symptoms ranged from back-ache to diabetes mellitus. A wide range of diseases are obviously prevalent in this area, many of which may be bacterial in origin given the high rate of enteric disease in this region. This may contribute not only to a reduced level of general health but also to the spread of resistant organisms.

Other factors such as diet, i.e. whether the volunteer was vegetarian or not, did not seem to make any difference. In the village of Melmoil, for instance, no vegetarians were reported but there was no apparent difference in the carriage of resistant organisms. The amount of meat eaten by each group was, however, very similar. Melmoil also had the lowest density of people per household; this also seemed to make little difference to the carriage rate of resistant organisms, although it is interesting to note that Melmoil did have only one person who carried an organism resistant to nalidixic acid. The average age or sex of the volunteers does not seem to be important; the average age of the urban group was higher than the rural groups (table 14).

Table 13. Symptoms and illnesses reported by volunteers before the collection of samples.

Location	No. of volunteers	Therapy*	No. reported symptoms	Diarrhoea	Cough	Fever	Abdominal pain	Vomiting	Dysuria	Miscell-aneous
Rural										
KVK	43	18	3	9	20	9	21	3	10	13
Kavanur	46	19	14	7	10	8	18	5	0	4
Melmoil	38	15	14	5	9	16	6	4	0	5
Combined	127	52	31	21	39	33	45	12	10	22
Urban										
Vellore	95	55	48	7	19	22	21	6	6	15

*This is the number of volunteers who had received therapy, other than antibiotics, in the week before sampling. Illnesses or symptoms had occurred in the previous month before sampling.

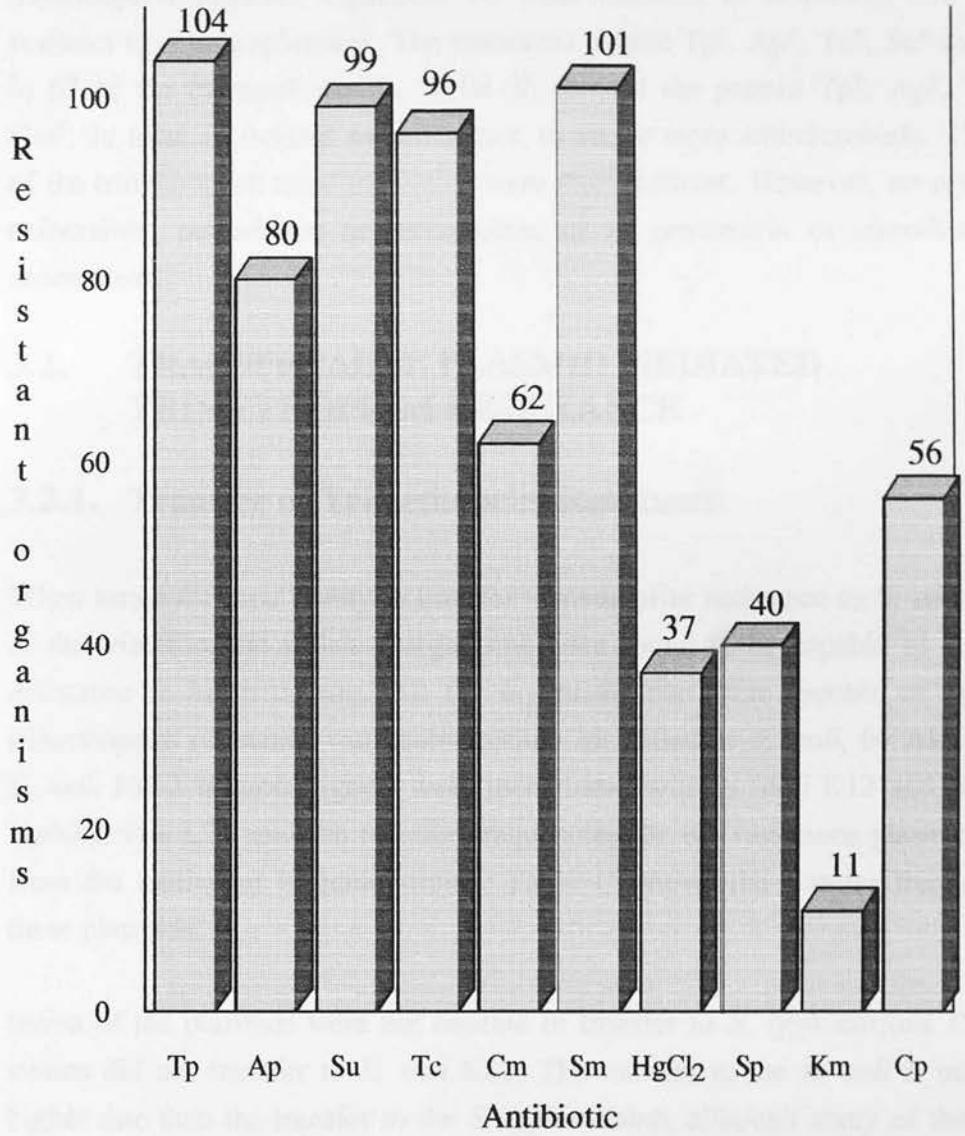
As part of the study the availability of antimicrobials in "over the counter sales" was assessed. It was ascertained that it was possible to buy many antimicrobials without prescription, except 2nd and 3rd generation cephalosporins but including the newer fluorinated 4-quinolones. Drugs that were freely available included trimethoprim, co-trimoxazole, ampicillin, cephalexin, norfloxacin, ciprofloxacin, tetracycline, chloramphenicol and erythromycin. These antimicrobials could be bought singly or in doses, thus there was no control over the dosing in those taking the drugs. Commensal flora and pathogens will be subject to challenge from antibacterials in uncontrolled self-dosing.

Table 14. Details of the population sample recorded in the questionnaires.

Location	Total	No. of males in group	Vegetarian	Mean age	No./ house	Meat/ month
Rural						
KVK	43	11	14	26.6	7.0	4.1
Kavanur	46	17	5	14.2	6.2	4.3
Melmoil	38	27	0	18.8	4.8	4.3
Combined	127	55	19	19.8	6.1	4.3
Urban						
Vellore	99	22	9	33.4	5.4	4.9

3.1.3. Resistance to Other Antimicrobials Amongst the Trimethoprim Resistant Isolates

Figure 3. Trimethoprim resistant organisms resistant to other antimicrobial agents.



104 trimethoprim resistant bacteria were studied in detail, only those capable of transferring trimethoprim resistance were subsequently identified. Almost all the trimethoprim resistance was high level, 96 of the strains were resistant to >1024mg/L. Of the other strains, one had an MIC of 256mg/L, five had an MIC of 64 mg/L, and two had an MIC of 32 mg/L.

Trimethoprim resistant organisms, isolated during the survey were shown, by breakpoint sensitivity testing, to be resistant to a wide range of other antimicrobial agents (figure 3). Almost all the organisms were also resistant to sulphamethoxazole (99), tetracycline (96) and streptomycin (101). Of the 104 trimethoprim resistant organisms 80 were resistant to ampicillin and 62 were resistant to chloramphenicol. The resistance pattern Tp^r, Ap^r, Tc^r, Su^r was present in 67 of the bacterial strains, while 56 showed the pattern Tp^r, Ap^r, Tc^r, Su^r, Cm^r. In total 78 isolates were resistant to six or more antimicrobials. Thus many of the trimethoprim resistant strains were multiresistant. However, no resistance to cefotaxime, ceftazidime or cefuroxime, or to gentamicin or ciprofloxacin was encountered.

3.2. TRANSFERABLE PLASMID MEDIATED TRIMETHOPRIM RESISTANCE

3.2.1. Transfer of Trimethoprim Resistance

When tested for their ability to transfer trimethoprim resistance to *E. coli* J62-2, 36 of the trimethoprim resistant organisms were found to be capable of transferring resistance in broth culture. All the organisms that were capable of transferring trimethoprim resistance were subsequently identified as *E. coli*, by API 20E. The *E. coli* J62-2 transconjugants were then mated with *E. coli* K12 and *Salmonella typhimurium* LT2 and the transfer frequencies for the resistance plasmids derived from the mating of isogenic strains. Table 15 shows the transfer frequencies for these plasmids.

Seven of the plasmids were not capable of transfer to *S. typhimurium*. One of the strains did not transfer to *E. coli* K12. The transfer to the *E. coli* is usually at a higher rate than the transfer to the *S. typhimurium*, although many of the plasmids transferred at similar frequencies to the *E. coli* as they did to the *S. typhimurium*. Thus, 29 of the 36 transferable plasmids encoding resistance to trimethoprim and other agents in normal faecal flora were at least capable of replication and expression of resistance in *S.typhimurium*, an enteric pathogen.

Table 15. The transfer frequencies of trimethoprim resistance plasmids.

Strain	Transfer frequency to *E. coli* K12	Transfer frequency to *S. typhimurium* LT2
2	3.7×10^{-5}	5.0×10^{-7}
6	8.2×10^{-4}	5.4×10^{-4}
7	4.8×10^{-4}	1.9×10^{-4}
9	1.3×10^{-5}	-
10	1.1×10^{-3}	7.3×10^{-9}
13	4.5×10^{-4}	2.0×10^{-4}
16	9.3×10^{-3}	3.1×10^{-9}
20	7.6×10^{-4}	1.9×10^{-4}
26	1.1×10^{-3}	5.5×10^{-4}
29	1.1×10^{-3}	5.6×10^{-4}
32	7.3×10^{-4}	1.5×10^{-4}
34	1.2×10^{-3}	4.0×10^{-7}
35	1.2×10^{-3}	7.9×10^{-9}
36	7.6×10^{-4}	2.5×10^{-9}
60	5.5×10^{-6}	-
64	5.8×10^{-7}	-
91	1.3×10^{-5}	2.2×10^{-4}
111	1.2×10^{-3}	8.9×10^{-6}
118	8.5×10^{-4}	2.8×10^{-4}
120	1.3×10^{-4}	8.5×10^{-7}
147	1.1×10^{-4}	1.1×10^{-4}
160	8.9×10^{-5}	-
163	1.2×10^{-3}	8.8×10^{-4}
166	-	-
167	1.2×10^{-3}	1.2×10^{-3}
179	1.1×10^{-3}	4.5×10^{-8}
185	3.0×10^{-4}	5.9×10^{-5}
186	1.0×10^{-5}	-
188	4.3×10^{-4}	-
190	5.1×10^{-4}	3.4×10^{-5}
192	5.4×10^{-4}	1.0×10^{-4}
198	5.0×10^{-4}	3.5×10^{-5}

Table 15. The transfer frequencies of trimethoprim resistance plasmids, continued.

Strain	Transfer frequency to *E. coli* K12	Transfer frequency to *S. typhimurium* LT2
199	7.4×10^{-6}	9.9×10^{-6}
208	1.2×10^{-3}	1.2×10^{-3}
217	1.2×10^{-3}	1.2×10^{-3}
219	1.3×10^{-3}	1.3×10^{-3}

- = No transfer observed.

3.2.2. MICs for Trimethoprim of the *E. coli* J62-2 Transconjugants

When the MICs of trimethoprim were derived for the transconjugants (table 16), it was shown that those organisms that had low level resistance in the wild-type conferred low level resistance in the transconjugant. Only one incidence of low level resistance to trimethoprim was recorded that was not transferable, strain 206. No wild-type strain possessed high level resistance but conferred low level resistance in the transconjugant. The other 29 transconjugants all demonstrated high level resistance to trimethoprim.

3.2.3. MICs of Other Antimicrobials in the Trimethoprim Resistant Transconjugants

MICs of ampicillin, sulphamethoxazole, amoxycillin/clavulanic acid, spectinomycin, streptomycin and tetracycline were determined (table 16). Breakpoint sensitivities were determined for chloramphenicol (8mg/L), kanamycin (8mg/L), cephaloridine (8mg/L) and mercuric chloride (50mg/L) (see tables of resistance profiles for resistance markers determined by "break-point"). None of the transconjugants were found to be resistant to gentamicin (2mg/L), cefotaxime (2mg/L), cephalexin (2mg/L), ceftazidime (mg/L), nalidixic acid (8mg/L) or ciprofloxacin (1mg/L).

Table 16. MICs of the *E. coli* J62-2 transconjugants.

Strain	Tp	Su	Ap	Sm	Tc	Sp	Amox/clav
2	512	>1024	1024	32	>128	8	16
6	512	256	128	64	>128	16	8
7	512	>1024	4	64	8	8	8
9	512	>1024	1024	32	>128	8	16
10	4	>1024	256	64	>128	8	8
13	512	<8	4	<4	8	8	4
16	4	>1024	256	64	>128	8	8
20	512	>1024	256	128	>128	8	8
26	512	256	256	128	>128	128	8
29	512	256	128	64	>128	16	8
32	512	>1024	4	32	8	8	4
34	4	>1024	256	64	>128	8	8
35	4	>1024	256	64	>128	8	8
36	4	>1024	256	64	>128	8	8
60	512	>1024	1024	32	>128	8	16
64	512	<8	4	<4	>128	8	8
91	512	<8	4	<4	8	8	8
111	512	<8	128	64	>128	8	8
118	16	>1024	128	128	8	8	8
120	512	>1024	256	64	>128	8	8
147	512	<8	4	<4	8	8	4
160	512	<8	128	<4	>128	8	8
163	512	>1024	128	64	8	16	4
166	512	<8	128	<4	8	8	8
167	512	256	128	64	>128	16	4
179	16	>1024	4	128	8	8	8
186	512	>1024	1024	32	>128	8	16
188	512	<8	4	64	>128	8	8
190	512	>1024	4	32	>128	8	8
192	512	<8	4	<4	8	8	8
198	512	>1024	256	128	8	8	8
199	512	>1024	128	64	>128	8	8
208	512	1024	128	64	>128	16	8

Table 16. MICs of the *E. coli* J62-2 transconjugants, continued.

Strain	Tp	Su	Ap	Sm	Tc	Sp	Amox/clav
217	512	>1024	128	64	>128	16	8
219	512	1024	128	64	>128	16	8

Twenty five of the transconjugants were found to be resistant to ampicillin; four of these were also found to be resistant to amoxycillin/clavulanic acid and cephaloridine and had high MICs of ampicillin, 1024mg/L.

Surprisingly, only seven strains were resistant to chloramphenicol. Six transconjugants were resistant to kanamycin. As might be expected, many of the transconjugants were also resistant to sulphamethoxazole; in total 25 were resistant to the combination of trimethoprim/sulphamethoxazole. There were 17 different antibiotic resistance patterns given by the transconjugants tested against these drugs (table 17). This suggested that at least 17 different plasmids were involved in the transfer of trimethoprim resistance in this population.

Table 17. Resistance profiles of the trimethoprim resistant transconjugants.

Resistance profile	Transconjugant
Tp	13, 91, 147, 185
Tp Tc	188
Tp Ap	166
Tp Su Sm	7, 32
Tp (L) Su Sm	179
Tp Tc Cm Hg	64
Tp Tc Cm Hg Ap	160
Tp Tc Cm Ap	111
Tp Su Tc Sm	190
Tp Su Tc Sm Ap	199
Tp(L) Su Tc Sm Ap	10, 16, 34, 35, 36
Tp(L) Su Sm Ap Km	118
Tp Tc Ap Sp Sm Km	163
Tp Su Tc Sm Ap	20, 120, 198
Tp Su Ap Sm Sp	217,
Tp Su Tc Ap Sm Sp	6, 26, 29, 167, 208,219
Tp Su Tc Cm Sm Ap Km Cp Hg Ag	186, 2, 9, 60

L indicates low level resistance to trimethoprim.

3.2.4. Restriction Endonuclease Digest Analysis of Transconjugant Plasmids

The plasmids responsible for transferable trimethoprim resistance were analysed by restriction endonuclease digestion. The plasmids were grouped according to antibiotic resistance pattern so that those sharing the same resistance profile could be more easily compared. The plasmids were digested with *Hin*dIII. 26 different plasmid types were distinguished by this method. This was later confirmed by further analysis and restriction endonuclease digestion during identification of the mechanisms responsible for trimethoprim resistance in these plasmids.

Thus trimethoprim resistance in the normal flora of this population was not the result of the spread of a few plasmids. Transferable trimethoprim resistance, accompanied by a range of other resistance mechanisms, was the result of the presence of different plasmids.

3.3. IDENTIFICATION OF DIHYDROFOLATE REDUCTASES RESPONSIBLE FOR TRIMETHOPRIM RESISTANCE

The mechanisms responsible for trimethoprim resistance in the transconjugants were identified by means of biotinylated *dhfr* gene probes as outlined in the Materials and Methods.

All of the plasmids were hybridized with all the probes listed in Materials and Methods. None of the plasmids were shown to possess more than one resistant DHFR. Eleven of the plasmids were demonstrated to harbour the *dhfr* type I, 18 were shown to harbour the *dhfr* type V and seven carried the *dhfr* type IV.

3.3.1. Plasmids Harbouring the Type I *dhfr* Gene

All of the plasmids that carried the type I *dhfr* (figure 4) also gave a positive signal when hybridized with the probe for the integrase-like ORF from Tn*7* (figure 5). This open reading frame is always associated with the transposon Tn*7*; thus all of the type I *dhfr* genes identified here were probably associated with transposon Tn*7*, the most frequently found location for this gene. Most of the plasmids that carried

the *dhfr* type I also carried spectinomycin resistance which is also normally associated with Tn*7*. There were three exceptions, 185 and 188 carried only trimethoprim resistance and 111, although it also harboured resistance to tetracycline, chloramphenicol and ampicillin, was sensitive to spectinomycin.

All of the remaining plasmids had very similar resistance profiles (table 18) but most had different restriction digest patterns. However, 6 and 26 had the same *Bam*H1 digest patterns (figure 6). The three plasmids 208, 217 and 219 were also very similar, although the presence of other smaller plasmids presented a confusing picture, 217 was not resistant to tetracycline and seemed to have a large section deleted; it was somewhat smaller than 208 and 219 (figure 6). The other plasmids were clearly distinguishable from one another. Several of the transconjugants harbour two large plasmids which made the task of distinguishing the digest patterns of the individual plasmids that much more difficult. The transconjugants 167 and 188 both carried two large plasmids. The presence of the *dhfr*I was confirmed in a hybridisation with digested plasmid DNA (figure 6 and 7).

Table 18. Characteristics of plasmids positive for the *dhfr*I probe.

Plasmid	Resistance profile	Plasmid molecular size (Kb)
185	Tp	40
188	Tp, Tc	60, 85
111	Tp, Tc, Cm, Ap	77, 2
6	Tp, Su, Tc, Ap, Sm, Sp	48, 4
26	Tp, Su, Tc, Ap, Sm, Sp	48, 4
29	Tp, Su, Tc, Ap, Sm, Sp	94
208	Tp, Su, Tc, Ap, Sm, Sp	57, 7, 4
217	Tp, Su, Ap, Sm, Sp	35, 7, 4
219	Tp, Su, Tc, Ap, Sm, Sp	57, 7, 4
167	Tp, Su, Tc, Ap, Sm, Sp	77, 30, 2
163	Tp, Km, Tc, Ap, Sm, Sp	84

Figure 4. Southern blot of plasmid DNA of transconjugants harbouring the *dhfr*I gene hybridised with the probe for the *dhfr*I gene.

(A) plasmid pUN433 carries the *dhfr*V and was included as a negative control; plasmid DNA of transconjugants (B) R483 carries the *dhfr*I gene; (C) R67 carries the *dhfr*II and is also included as a negative control; (D) 219; (E) 217; (F) 208; (G) 188; (H) 185; (I) 167; (J) 163; (K) 111; (L) 29; (M) 26; (N) 6.

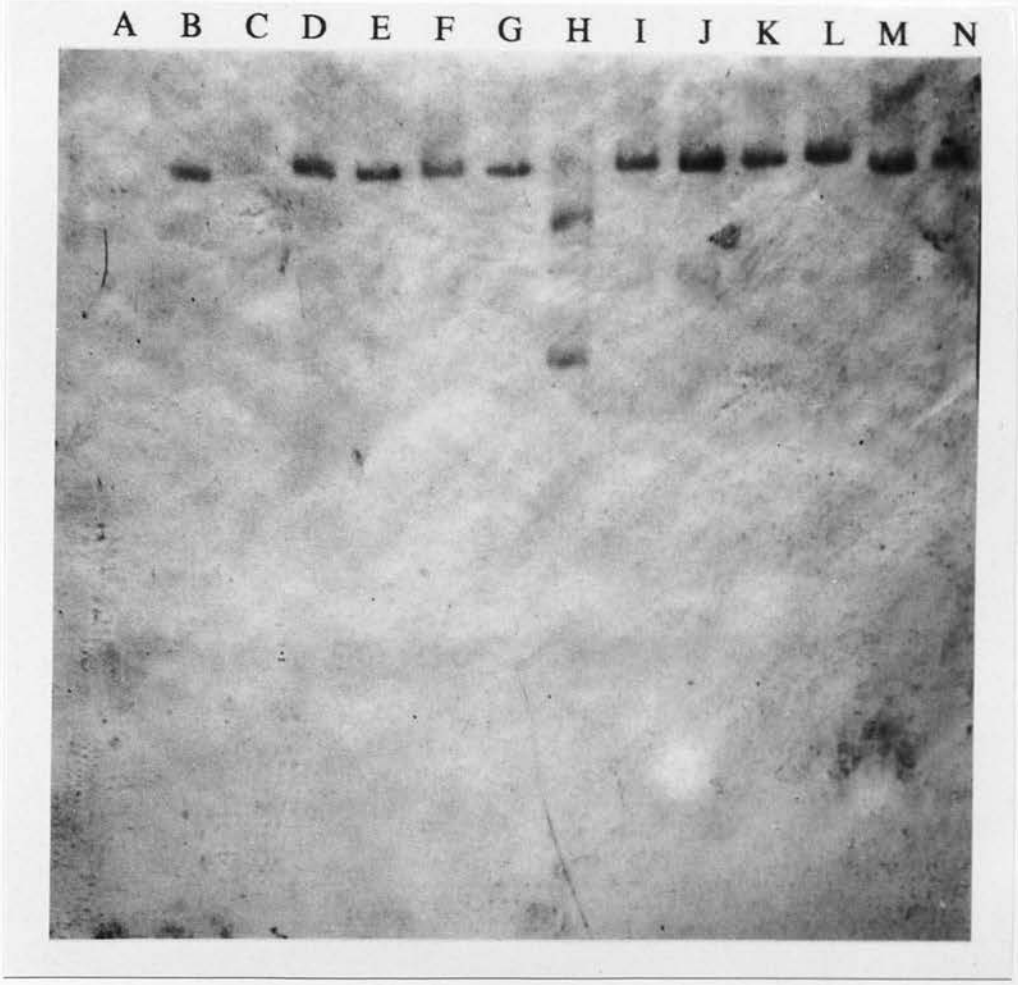


Figure 5. Southern blot of plasmid DNA of transconjugants harbouring the *dhfr*I gene hybridised with the probe for the Tn*7* integrase-like ORF.

Tracks are (A) pUN1053 is a recombinant carrying the *dhfr*VII; (B) pAZ1is a plasmid carrying the *dhfr*III, which is not associated with a transposon; (C) R483 carries the *dhfr*I gene; (D) R751 carries the *dhfr*II and was also included as a negative control; (E) R67 carries the *dhfr*II and is also included as a negative control; (F) 219; (G) 217; (H) 208; (I) 188; (J) 185; (K) 167; (L) 163; (M) 111; (N) 29; (O) 26; (P) 6,

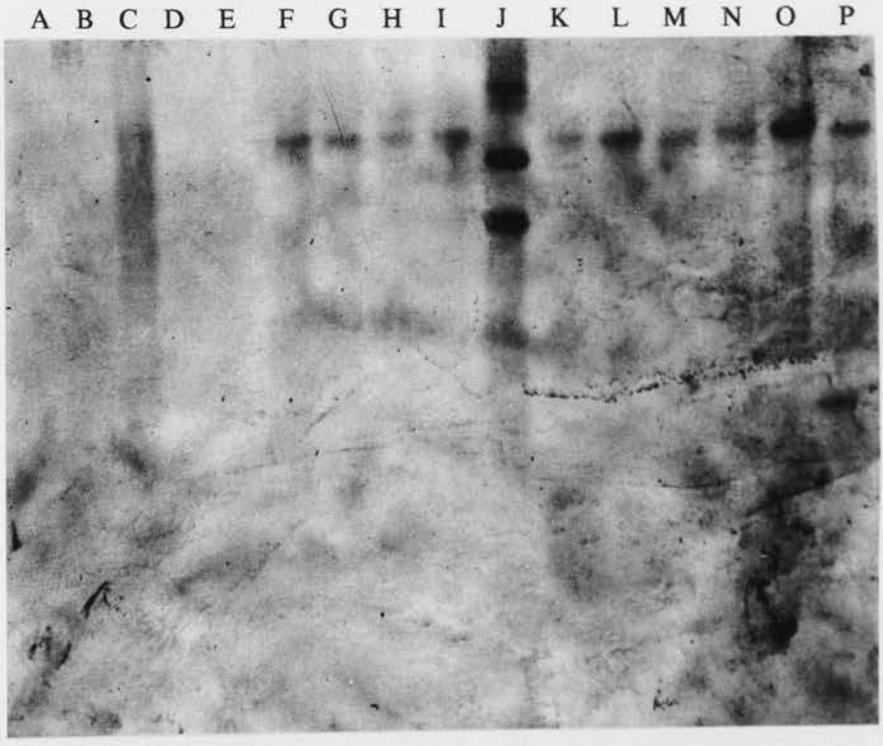


Figure 6. A 0.8% agarose gel of a *Bam*H1 restriction endonuclease digest of plasmid DNA from transconjugants harbouring the *dhfr*I gene.

Tracks are of plasmid DNA from transconjugants (A) 6; (B) 26; (C) 29; (D) 111; (E) 163; (F) 167; (G) 185; (H) 188; (I) 208; (J) 217; (K) 219; (L) plasmid pUN433 carries the *dhfr*V and was included as a negative control; (M) λ DNA *Hin*dIII marker.

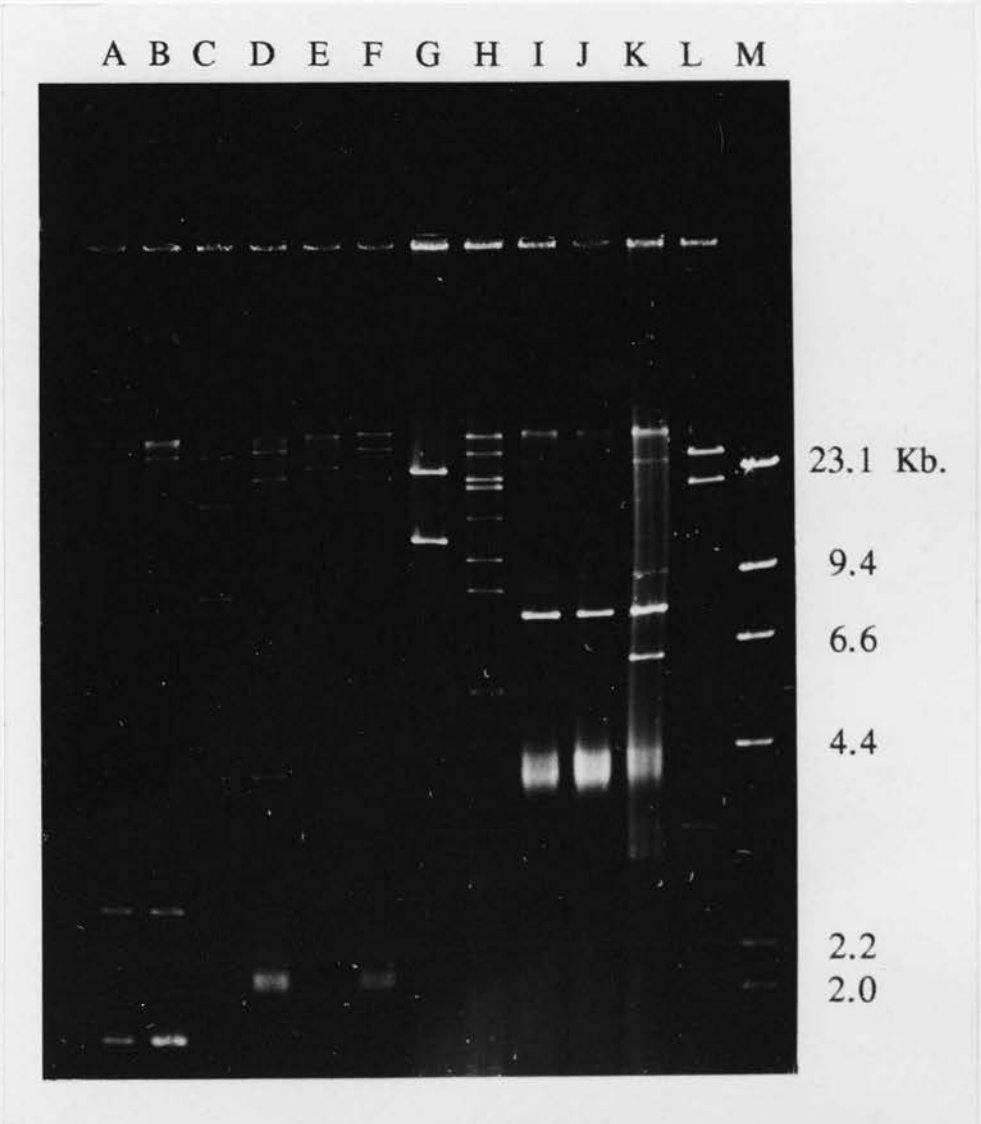
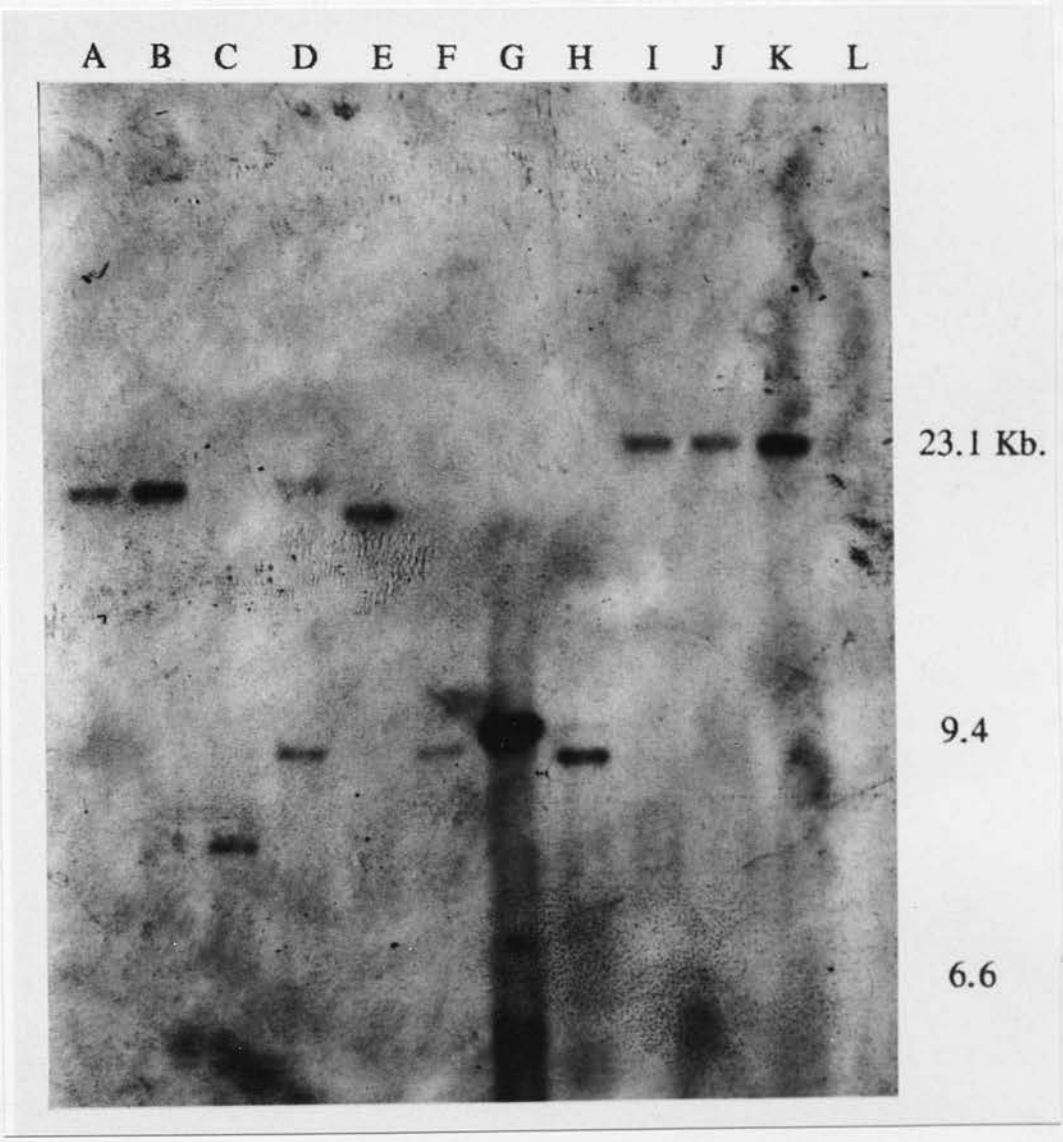


Figure 7. Southern blot of a *Bam*H1 restriction endonuclease digest of plasmid DNA of transconjugants harbouring the *dhfr*I gene hybridised with the probe for the *dhfr*I gene.

Tracks are digested plasmid DNA of transconjugants (A) 219; (B) 217; (C) 208; (D) 188; (E) 185; (F) 167; (G) 163; (H) 111; (I) 29; (J) 26; (K) 6; (L) plasmid pUN433, carries the *dhfr*V and was included as a negative control.



3.3.2. Plasmids Harbouring the Type V *dhfr* Gene

Many of the 18 plasmids carrying the *dhfr* V (figures 8 and 9) were very large and were responsible for a number of resistance mechanisms other than trimethoprim (table 19). The presence of the gene was confirmed by hybridisation of the probe with plasmid DNA subjected to restriction endonuclease digestion (figures 10, 11, 12 and 13). The digest patterns also served to distinguish plasmids that had similar antibiotic resistance profiles (figures 10 and 12). The transconjugants of the strains 13, 91, 147 and 192 all shared similar endonuclease digest patterns (figure 14), and were all resistant to trimethoprim only (table 19). They were, however, isolated from different areas of the study; 13 was from a strain isolated in Kilvayattarrankuppam, 91 from Kavanur, and 192 and 147 from the town of Vellore. The plasmids of 91 and 147, although isolated in different areas, had digest patterns plasmids which seemed almost identical (figure 14).

The same antibiotic resistance pattern was also shared by 7 and 32; they also had similar digest patterns, although they were not identical (figure 15). The preparation and digestion of plasmids 9 and 60 was unreliable, probably because of their large size. Although they had the same antibiotic resistance profile, they were not identical (figure 15). They did, however, share a number of common bands when subjected to restriction endonuclease digestion. Again the presence of more than one large plasmid in the transconjugant presented a confusing picture in the digest patterns (especially in strains 64, 160 and 186).

Of the plasmids that possessed the *tnp*A gene, all but two had sulphonamide resistance associated with them. These two plasmids, 64 and 160, were resistant to mercuric chloride, another marker commonly associated with the Tn*21*-like transposons. Four other plasmids also have this marker; these were the "multi-resistant" plasmids that were resistant to several antibiotics, including kanamycin, amoxycillin/clavulanic acid and cephaloridine (table 20).

Table 19. Characteristics of plasmids positive for the *dhfr*V probe.

Donor strain	Resistance profile	Plasmid molecular size (Kb)
13	Tp	69
91	Tp	46, 2
147	Tp	53, 13, 4
192	Tp	54, 8, 3
166	Tp Ap	64, 13, 6
7	Tp, Su, Sm	82
32	Tp, Su, Sm	74, 17, 4, 2
64	Tp, Tc, Cm, Hg	80, 55
190	Tp, Su, Tc, Sm	100, 8, 3
160	Tp, Tc, Cm, Ap, Hg	80, 46
199	Tp, Su, Tc, Sm, Ap	82
20	Tp, Su, Tc, Sm, Ap	120
120	Tp, Su, Tc, Sm, Ap	30
198	Tp, Su, Tc, Sm, Ap	52
186	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	70, 63
2	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	89
9	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	100
60	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	108

Seven of the plasmids carrying the *dhfr* type V did not have sulphamethoxazole resistance associated with them (table 19). Sulphamethoxazole resistance is usually found associated with the *dhfr* type V as part of Tn*21*. All of the plasmids harbouring the *dhfr*V gave a positive signal when hybridized with the integrase-like ORF of Tn*21*, which indicated the presence of the integrase system commonly found associated with this gene (figure 16). In eight of the plasmids, however, there was no hybridisation with the probe for the *tnp*A gene of Tn*21* (figures 17 and 18). This would seem to indicate the lack of a complete and functional transposon. The *tnp*A region may have been deleted or the gene has been inserted in the plasmid, by the action of the integrase system, independent of a transposon intermediary (information on hybridisation with the transposon probes is summarized in table 20). This would seem likely in those five plasmids that did not have sulphonamide resistance associated with them and were negative for the *tnp*A

probe, sulphonamide resistance being associated with the Tn*21*-like transposons (table 20). Four of these plasmids mediated only trimethoprim resistance, whilst the fifth mediated only trimethoprim and ampicillin resistance .

Table 20. Results of hybridization of plasmids positive for the *dhfr*V probe with the probes for the integrase-like ORF of Tn*21*(In-Tn*21*) and the *tnp*A region of Tn*21* (*tnp*A).

Donor strain	Resistance profile	In-Tn*21*	*tnp*A
13	Tp	+	-
91	Tp	+	-
147	Tp	+	-
192	Tp	+	-
166	Tp, Ap	+	-
7	Tp, Su, Sm	+	-
32	Tp, Su, Sm	+	-
64	Tp, Tc, Cm, Hg	+	+
190	Tp, Su, Tc, Cm, Sm	+	-
160	Tp, Tc, Cm, Ap, Hg	+	+
199	Tp, Su, Tc, Sm, Ap	+	+
20	Tp, Su, Tc, Sm, Ap, Cp	+	+
120	Tp, Su, Tc, Sm, Ap, Cp	+	+
198	Tp, Su, Tc, Sm, Ap, Cp	+	+
186	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	+	+
2	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	+	+
9	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	+	+
60	Tp, Su, Tc, Cm, Sm, Ap, Cp, Hg, Km, Ag	+	+

Figure 9. Southern blot of plasmids showing positive hybridisation with the DNA probe for the *dhfr*V gene.

Tracks are (A) pUN433, plasmid carrying the *dhfr*V gene. Plasmid DNA from the strains (B) 91; (C) 64; (D) 60; (E) 132; (F) 20; (G) 13; (H) 9; (I) 7; (J) 2.

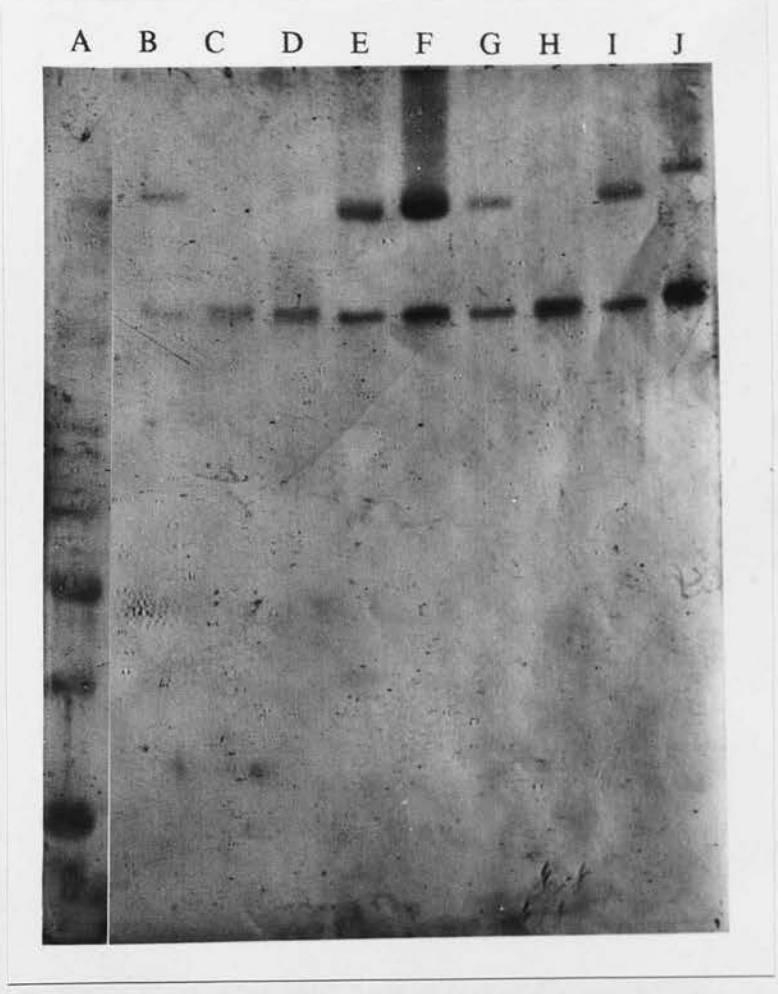


Figure 10. *Eco*R1 restriction endonuclease digest of plasmid DNA from the transconjugants harbouring the *dhfr*V gene.

*Eco*R1 digests of plasmid DNA from transconjugants (A) 13; (B) 91; (C) 147; (D) 166; (E) 192; (F) 7; (G) 32; (H) 64; (I) 190; (J) 160; (K) 199; (L) 20; (M) 120; (N) 198; (O) 186; (P) 2; (Q) 9; (R) 60; (S) λ DNA *Hin*dIII digest marker.

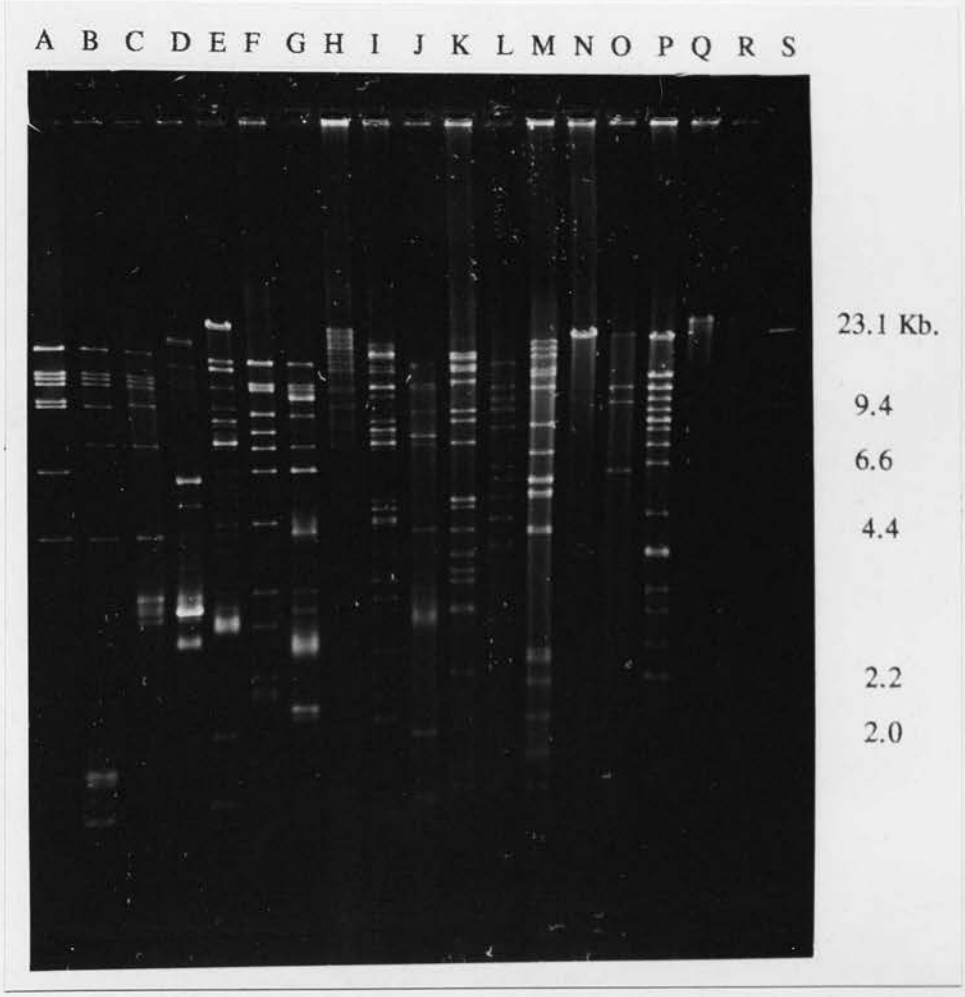


Figure 11. Southern blot of *Eco*R1 restriction endonuclease digest of plasmid DNA from the transconjugants harbouring the *dhfr*V gene hybridised with the probe for the *dhfr*V.

Tracks are *Eco*R1 digests of plasmid DNA from transconjugants hybridised with the *dhfr*V probe (A) 60; (B) 9; (C) 2; (D) 186; (E) 198; (F) 120; (G) 20; (H) 199; (I) 160; (J) 190; (K) 64; (L) 32; (M) 7; (N) 192; (O) 166; (P) 147; (Q) 91; (R) 13.

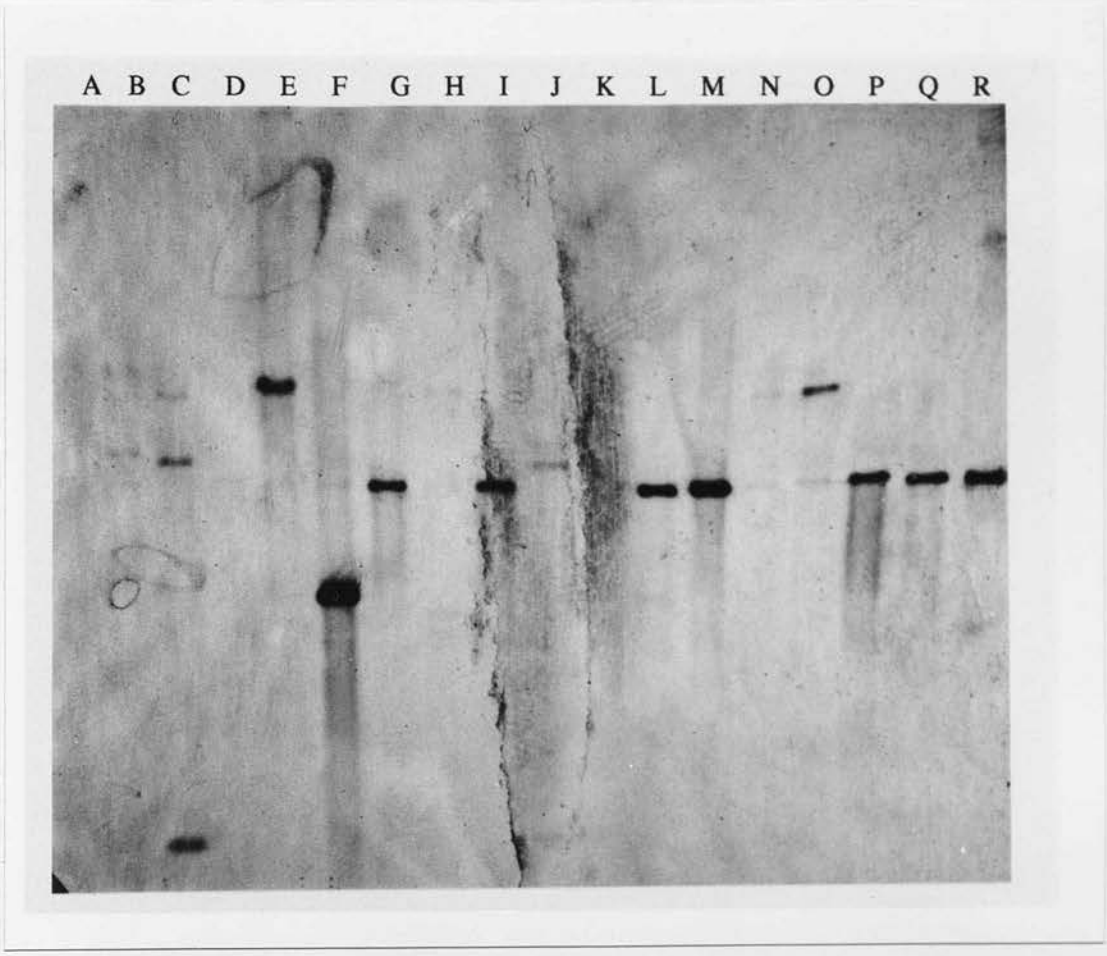


Figure 12. 0.8% agarose gel of the *Eco*R1 digests of plasmid DNA from transconjugants not shown in figure 10.

Tracks are *Eco*R1 digests of plasmid DNA from transconjugants (A) 64; (B) 190; (C) 160; (D) 199; (E) 20; (F) 120; (G) 198; (H) 186; (I) 2; (J) 9; (K) 60; (L) 64.

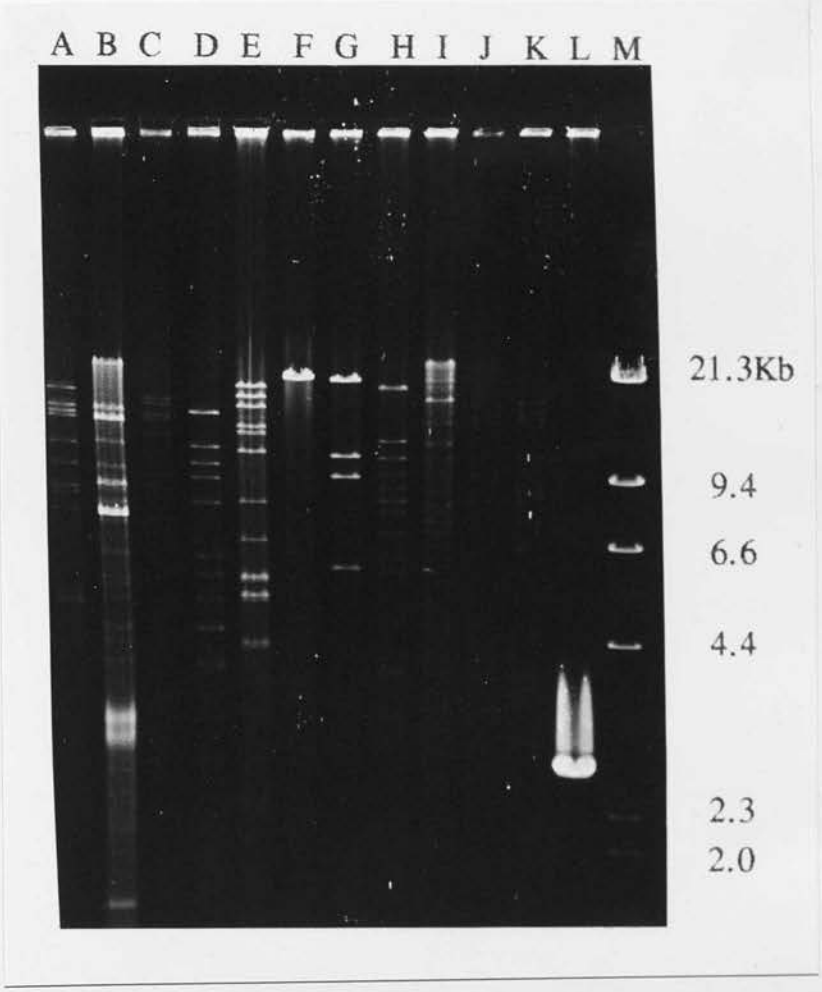


Figure 13. Southern blot of digests shown in figure 11, hybridised with the *dhfr*V probe.

(A) pUN433, plasmid carrying the *dhfr*V. *Eco*R1 digests of plasmid DNA from transconjugants hybridised with the *dhfr*V probe (B) 60; (C) 9; (D) 2; (E) 186; (F) 198; (G) 120; (H) 20; (I) 199; (J) 160; (K) 190; (L) 64.

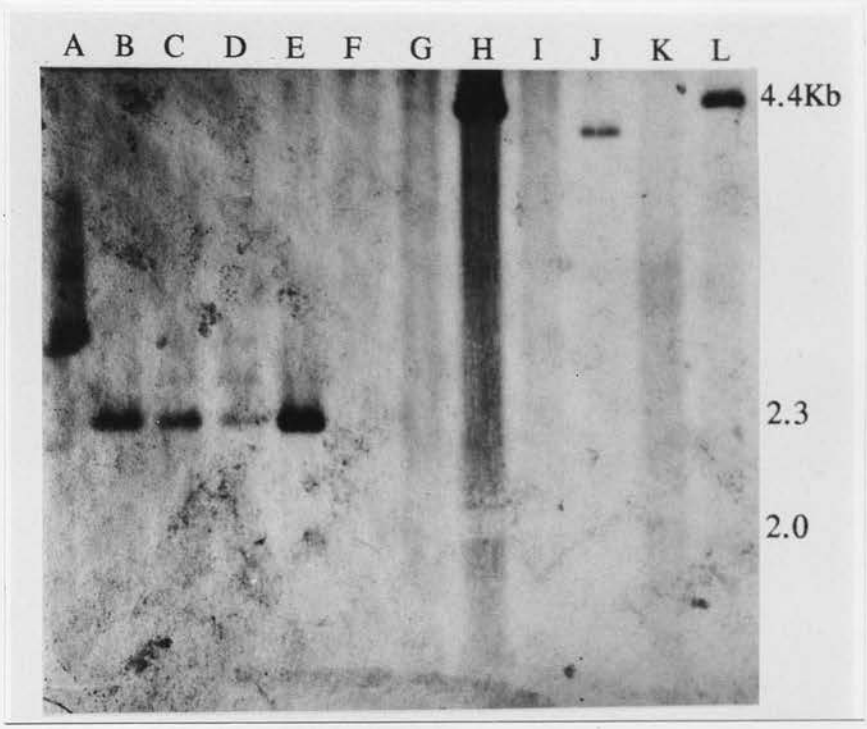


Figure 14. A 0.8% agarose gel of a *Bam*H1 restriction endonuclease digest of plasmid DNA from the transconjugants of 13, 91,147, and 192, illustrating the similarities between these plasmids.

Tracks are plasmid DNA from transconjugants digested with *Bam*H1. (A) 13; (B) 91; (C) 147; (D) 192.

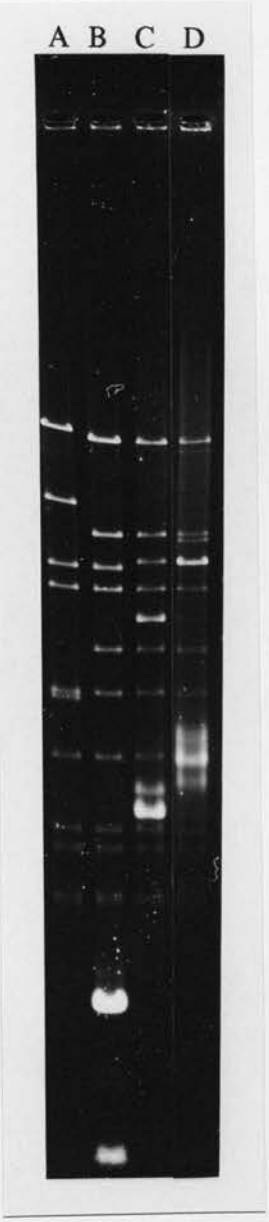


Figure 15. A 0.8% agarose gel of an *Eco*R1 restriction endonuclease digest of plasmid DNA from the transconjugants of 7, 32, 9 and 60, illustrating the similarities between 7 and 32; and 9 and 60.

Tracks are of plasmid DNA of transconjugants digested with *Eco*R1. (A) 7; (B) 32; (C) 9; (D) 60.

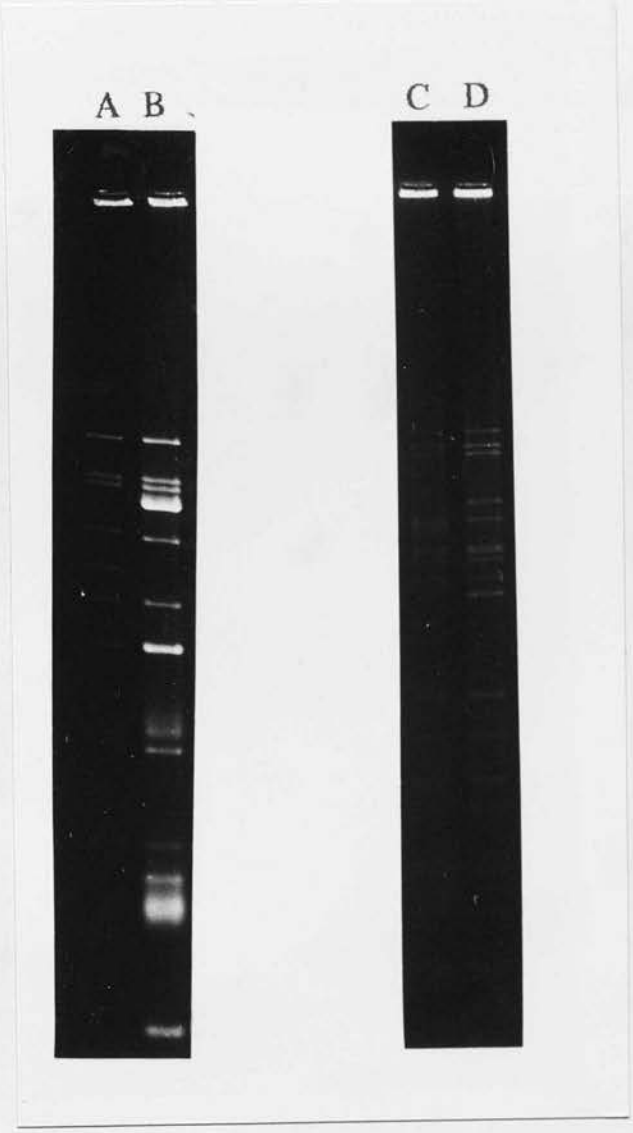


Figure 16. Southern blot of plasmids showing positive hybridisation with the DNA probe for the *dhfr*V gene hybridised with the probe for the Tn*21* integrase-like ORF.

Tracks are transconjugant plasmid DNA carrying the *dhfr*V gene, hybridised with the probe for the Tn*21* integrase-like ORF. Control plasmids (A) R483, plasmid carrying Tn*7*; (B) pUN433; (C) 198; (D) 196; (E) 192; (F) 190; (G) 186; (H) 166; (I) 160; (J) 147; (K) 120; (L) 91; (M) 64; (N) 60; (O) 32; (P) 20; (Q) 13; (R) 9; (S) 7; (T) 2.

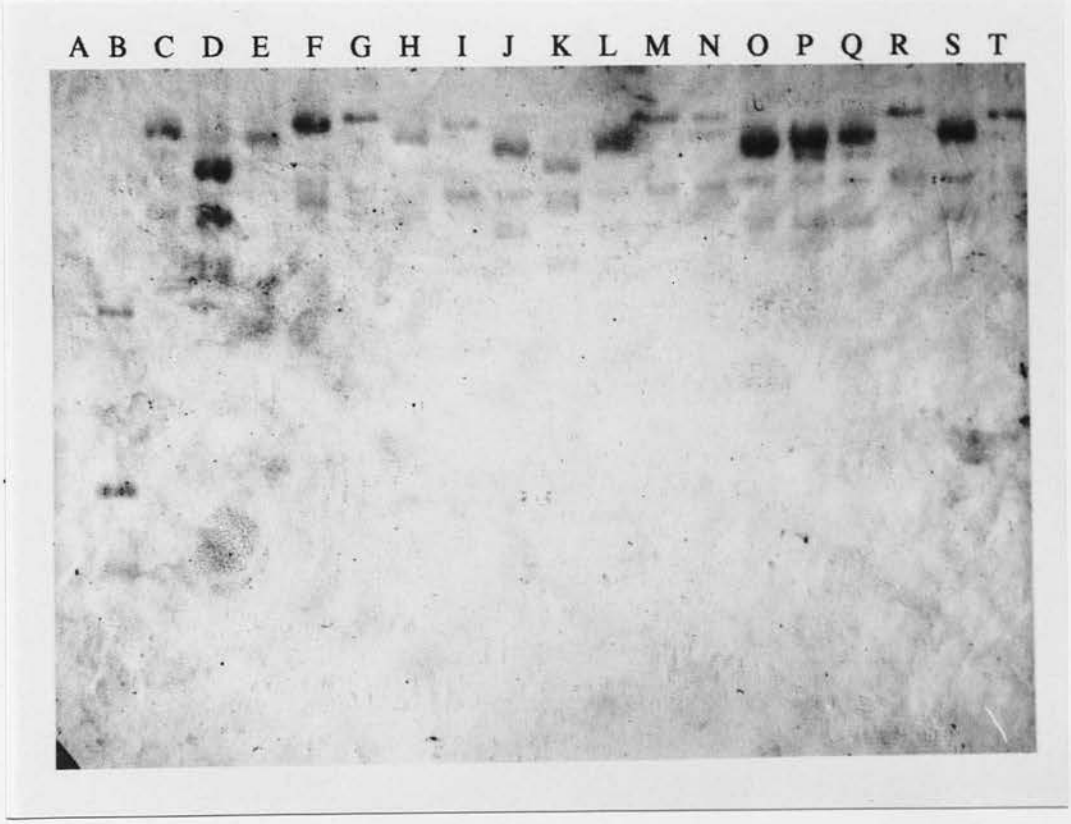


Figure 17. Southern blot of plasmids showing positive hybridisation with the DNA probe for the *dhfr*V gene hybridised with the probe for the Tn21 *tnp*A gene.

Tracks are transconjugant plasmid DNA of (A) pUN1053 a recombinant carrying the *dhfr*VII; (B) pUN433; (C) R483, plasmid carrying Tn*7*; (D) 199; (E) 198; (F) 192; (G) 190; (H) 186; (I) 166; (J) 160; (K) 147; (L) 120.

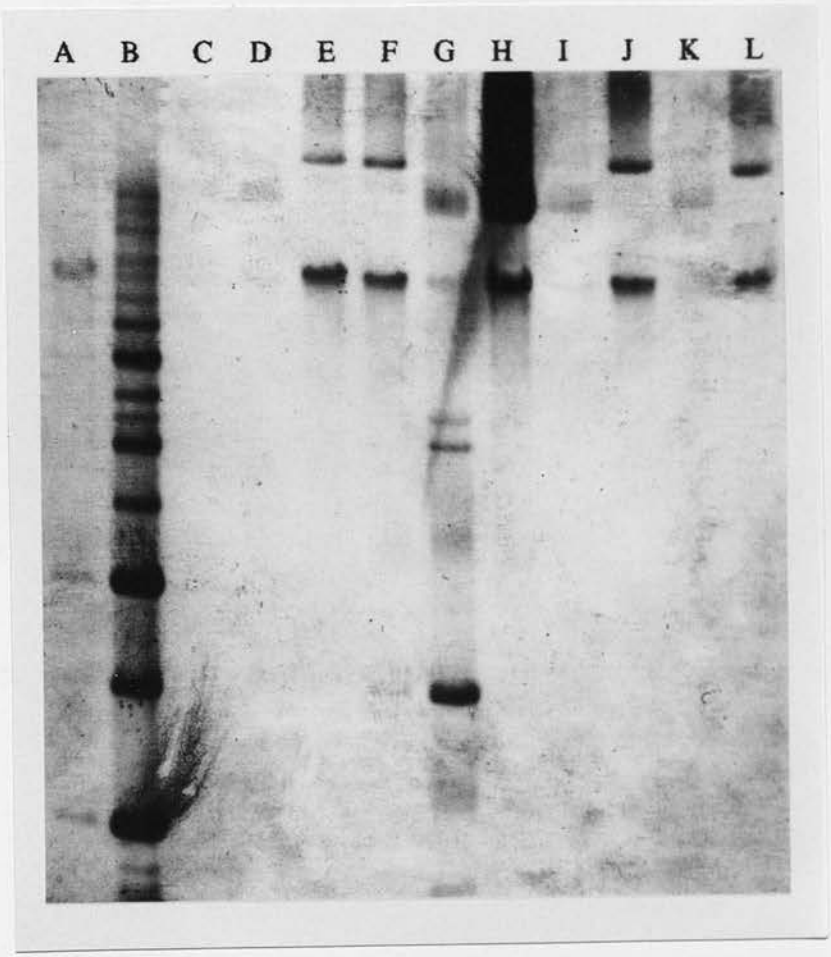


Figure 18. Southern blot of plasmids showing positive hybridisation with the DNA probe for the *dhfr*V gene hybridised with the probe for the Tn*21tnp*A gene.

Tracks are transconjugant plasmid DNA from (A) pUN1053 a recombinant carrying the *dhfr*VII; (B) pUN433; (C) R483, plasmid carrying Tn*7*; (D) 91; (E) 64; (F) 60; (G) 32; (H) 20; (I) 13; (J) 9; (K) 7; (L) 2.

3.3.3. Identification of *dhfr*I and *dhfr*V Genes in Wild-Type Strains Unable to Transfer Trimethoprim Resistance

Those strains that were found not to be able to transfer resistance to trimethoprim were tested for the presence of the *dhfr*I and *dhfr*V genes by colony blots and hybridisation with the probes for these genes labelled with ^{32}P.

The resistance profiles of the strains that showed positive hybridisation with the probes are shown in tables 21 and 22. In total 27 strains, that were not capable of the transfer of trimethoprim resistance, gave positive signals when hybridised with the *dhfr*I probe. In most of these strains it is likely that the *dhfr*I gene is located on the chromosome having been transferred to this location as part of the transposon Tn*7*. In 24 of these strains spectinomycin/streptomycin resistance was also present; this is a commonly found resistance marker associated with Tn*7*. Only three of the strains that showed positive hybridisation with the *dhfr*I gene were not resistant to spectinomycin/streptomycin; this may be the result of loss of this resistance gene or the presence of the *dhfr*I gene on a genetic element other than Tn*7*. There were 11 different resistance profiles given by those organisms positive for the *dhfr*I gene; however, 12 of the strains had one of these, and six had another (table 21).

In those strains that were not capable of transferring trimethoprim resistance, only four demonstrated positive hybridisation with the *dhfr*V probe. Three of these had the same resistance profile (table 22)

Thus the *dhfr* type I is found very much more frequently in non-transferable trimethoprim resistance, as opposed to the *dhfr* type V which in this survey was responsible for 50% of the transferable trimethoprim resistance.

Table 21. Strains that demonstrated positive hybridisation with the *dhfr*I gene probe but were not capable of transferring trimethoprim resistance.

Resistance profile	Strain number
Tp Su Ap Tc Cm Sm/Sp Hg Km	174
Tp Su Ap Tc Sm/Sp Hg Km	203
Tp Su Ap Tc Cm Sm/Sp	47, 54, 110, 112, 117, 141, 148, 152, 153, 161, 170, 193
Tp Su Ap Tc Cm Km	149
Tp Su Ap Cm Sm/Sp	195
Tp Su Ap Tc Cm	173
Tp Ap Tc Sm/Sp	45
Tp Su Tc Sm/Sp	61, 139, 155, 172, 194, 201
Tp Su Sm/Sp	162, 171
Tp	136

Table 22. Strains that demonstrated positive hybridisation with the *dhfr*V gene probe but did not transfer trimethoprim resistance.

Resistance profile	Strain number
Tp Su Ap Tc Sm	23
Tp Su Ap Tc Cm Sm Hg	37, 56, 191

3.4. IDENTIFICATION OF THE β-LACTAMASES MEDIATING RESISTANCE IN THE AMPICILLIN RESISTANT TRANSCONJUGANTS

Those transconjugants that exhibited resistance to ampicillin were tested for the presence of plasmid-mediated β-lactamases by iso-electric focusing in polyacrylamide gels on pH gradient of 3.5-10 (figures 19-21), followed by detection of the β-lactamases by staining with nitrocefin.

This procedure revealed that the presence of the β-lactamase TEM-1 was responsible for ampicillin resistance in all 25 of those transconjugants that exhibitted resistance to this drug. This included those transconjugants that were resistant to cephaloridine (20, 120, and 198), and those resistant to cephaloridine and amoxycillin/clavulanic acid (186, 2, 9 and 60).

Figure 19. Iso-electric focusing polyacrylamide gel (pI 3.5-10) of β-lactamases conferring ampicillin resistance on trimethoprim resistant transconjugants.

Tracks are β-lactamase preparations from the transconjugants of (A) 198; (B) 2; (C) 6; (D) 9; (E) 10; (F) 16; (G) 20; (H) 26; (I) 29; (J) 34; with (K) TEM-1(pI 5.4) and (L) SHV-1(pI 7.6), included as controls.

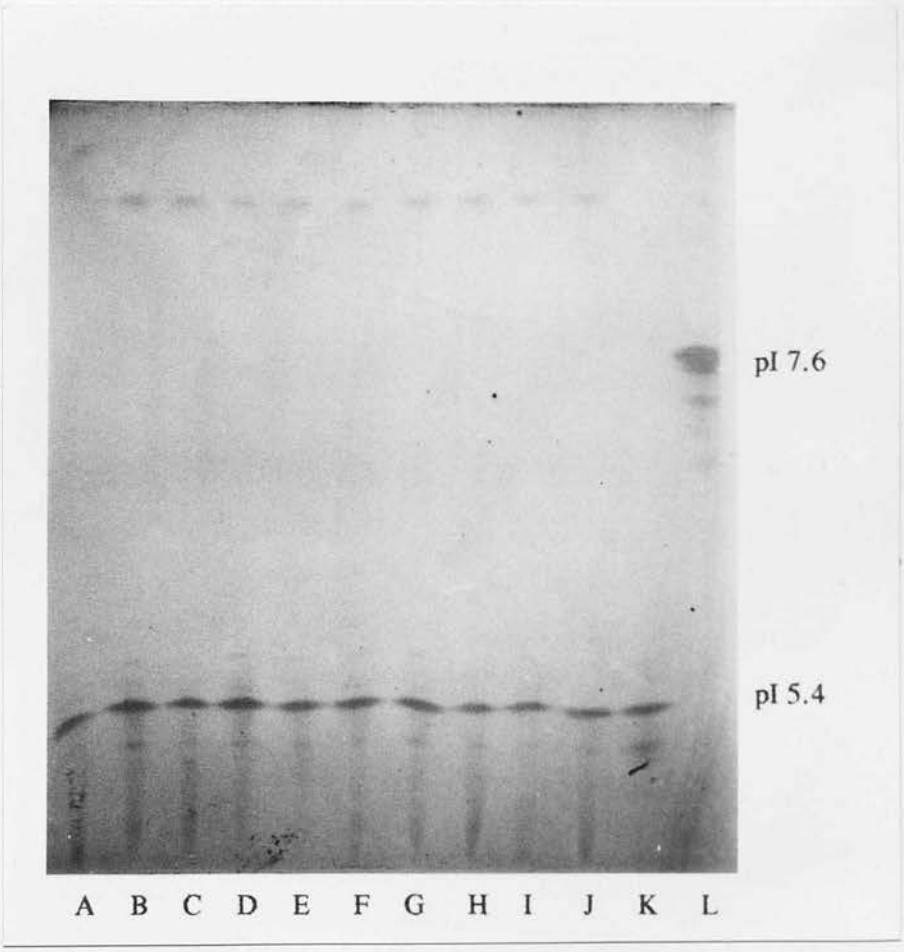


Figure 20. Iso-electric focusing polyacrylamide gel (pI 3.5-10) of β-lactamases conferring ampicillin resistance on trimethoprim resistant transconjugants.

Tracks are β-lactamase preparations from the transconjugants of (A) 35; (B) 36; (C) 60; (D) 111; (E) 118; (F) 120; (G) 160; (H) 163; (I) 166; with (J) SHV-1(pI 7.6), included as a control.

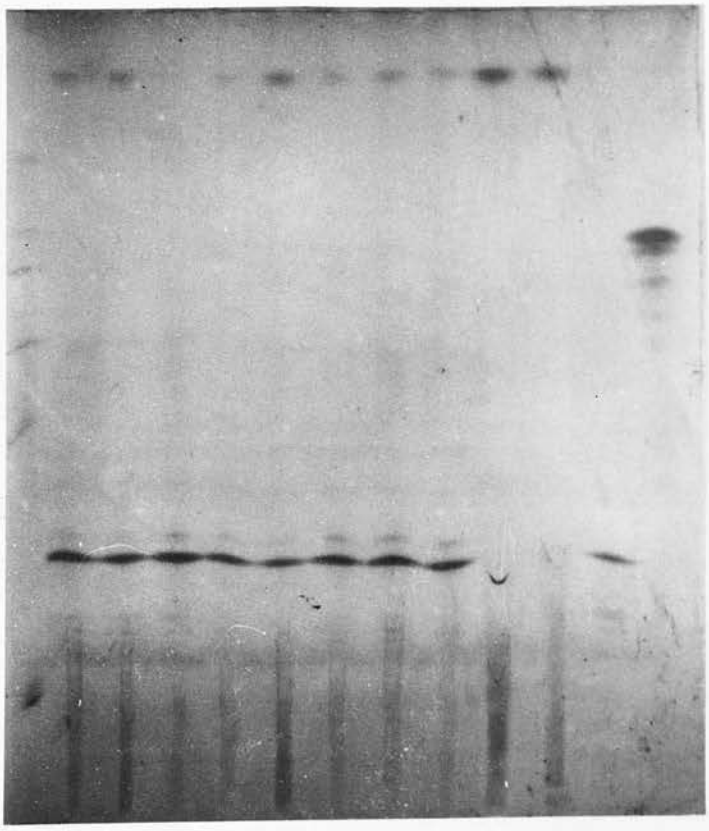
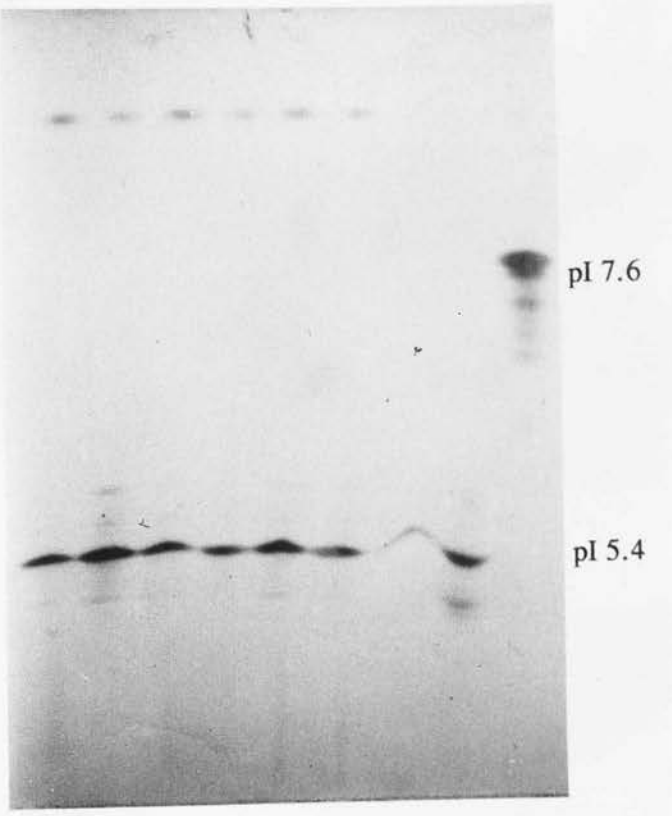


Figure 21. Iso-electric focusing polyacrylamide gel of (pI 3.5-10) β-lactamases conferring ampicillin resistance on trimethoprim resistant transconjugants.

Tracks are β-lactamase preparations from the transconjugants of (A) 167; (B) 186; (C) 199; (D) 208; (E) 217; (F) 219; (G) TEM-1 (pI 5.4) and (H) SHV-1 (pI 7.6), included as controls.



CHAPTER 4

THE TYPE IV DIHYDROFOLATE REDUCTASE

The *dhfr*IV gene confers only low level resistance to trimethoprim, 4 mg/L as determined on IST agar, yet it has persisted in this region and has not been detected elsewhere. In this area it represented about 20% of the transferable trimethoprim resistance. Thus, despite the presence of ostensibly more efficient resistance mechanisms, the *dhfr*I and V, the *dhfr*IV continues to be responsible for mediating resistance to trimethoprim. The properties of this enzyme were, therefore, investigated further

4.1. CHARACTERIZATION OF THE DHFR TYPE IV ISOLATED IN THE PRESENT STUDY

4.1.1. Plasmids that Harboured the Type IV *dhfr* Gene

Of those plasmids isolated that harbour the type IV *dhfr* (figure 22) five were identical in their restriction digest patterns (figures 23 and 25) and antibiotic resistance profile, 10, 16, 34, 35, 36 (table 23). All of these originated in the same village. The other two, one from the village of Melmoil furthest from the town and one from Vellore itself, had different antibiotic resistance profiles and restriction endonuclease digest patterns (figures 23 and 25). The plasmid of 179 lacked ampicillin resistance, and that of 118 had kanamycin resistance but lacked tetracycline resistance (table 23).

These plasmids were not only compared with each other but were also compared with those plasmids carrying the type IV *dhfr* from the study conducted in 1984 when they were first isolated (Young *et al* 1986a). There were some similarities

between these plasmids and those isolated in 1989, both in resistance profiles and molecular size (table 23).

When probed with the *Cla*I (1.4 Kb) probe for the type IV gene multiple signals were given in both sets of plasmids and in the plasmid from which the probe was originally derived. This was seen in digests of the sample plasmids with both *Cla*I (figure 24) and *Eco*RI (figure 26). This would seem to indicate the presence of repeated sequences on the plasmids.

Table 23. Plasmids positive for the *dhfr*IV probe in this study, and those plasmids isolated in 1984.

Plasmids	Resistance profile	Plasmid molecular size (Kb)
Plasmids isolated in 1989		
pUK2005 (10)	Tp, Su, Tc, Sm, Ap	65
pUK2007 (16)	Tp, Su, Tc, Sm, Ap	65
pUK2012 (34)	Tp, Su, Tc, Sm, Ap	65
pUK2013 (35)	Tp, Su, Tc, Sm, Ap	65
pUK2014 (36)	Tp, Su, Tc, Sm, Ap	65
pUK2019 (118)	Tp, Su, Sm, Ap, Km	52
pUK2026 (179)	Tp, Su, Sm	77
Plasmids isolated in 1984		
pUK1123	Tp, Su, Sm	78
pUK1115	Tp, Su, Sm, Km	81
pUK1119	Tp, Su, Sm, Ap, Km	79
pUK1115	Tp, Su, Sm, Ap, Tc, Cm	89
pUK1109	Tp, Su, Sm, Ap, Tc, Cm	99
pUK1118	Tp, Su, Sm, Ap, Tc, Cm, Km	100
pUK1114	Tp, Su, Sm, Ap, Tc, Cm, Km	122

Numbers in parentheses are the original donor strains.

Figure 22. Southern blot of plasmids harbouring the *dhfr*IV gene, hybridised with the DNA probe for the *dhfr*IV gene.

Tracks are transconjugant plasmid DNA of; (numbers in parentheses indicate original donor strain). (A) pUK2005 (10); (B) pUK2007 (16); (C) pUK2012 (34); (D) pUK2013 (35); (E) pUK2014 (36); (F) pUK2019 (118); (G) pUK2026 (179); (H) pUK1123; (I) R751; (J) pAZ1.

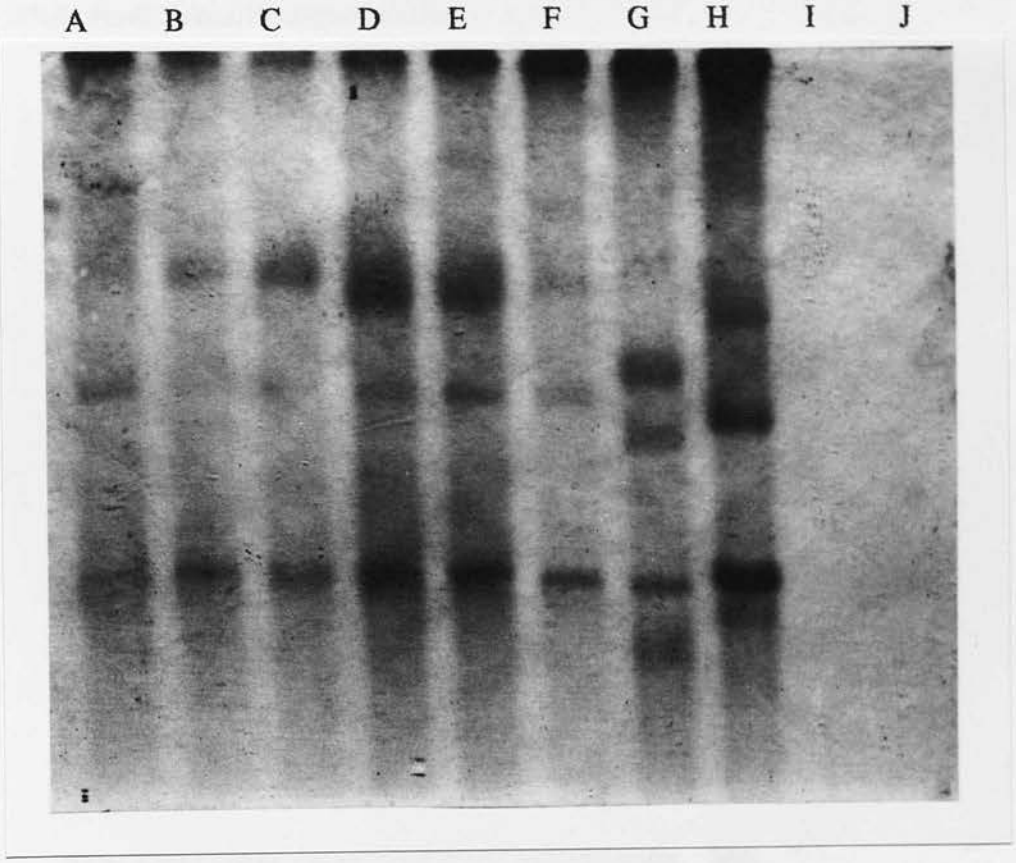


Figure 23. A 0.8% agarose gel of *Cla*1 restriction endonuclease digests of plasmids harbouring the *dhfr*IV gene from this study (1989) and the study by Young *et al* (1986a) (pUK1109-pUK1123).

Tracks are transconjugant plasmid DNA digested with *Cla*1, (numbers in parentheses indicate original donor strain). (A) pUK2005 (10); (B) pUK2007 (16); (C) pUK2012 (34); (D) pUK2013 (35); (E) pUK2014 (36); (F) pUK2019 (118); (G) pUK2026 (179); (H) pUK1109; (I) pUK1114; (K) pUK1115; (L) pUK1116; (M) pUK1118; (N) pUK1119; (O) pUK1123; (P) *dhfr*IV probe fragment; (Q) λ DNA *Eco*R1/*Hin*dIII digest marker.

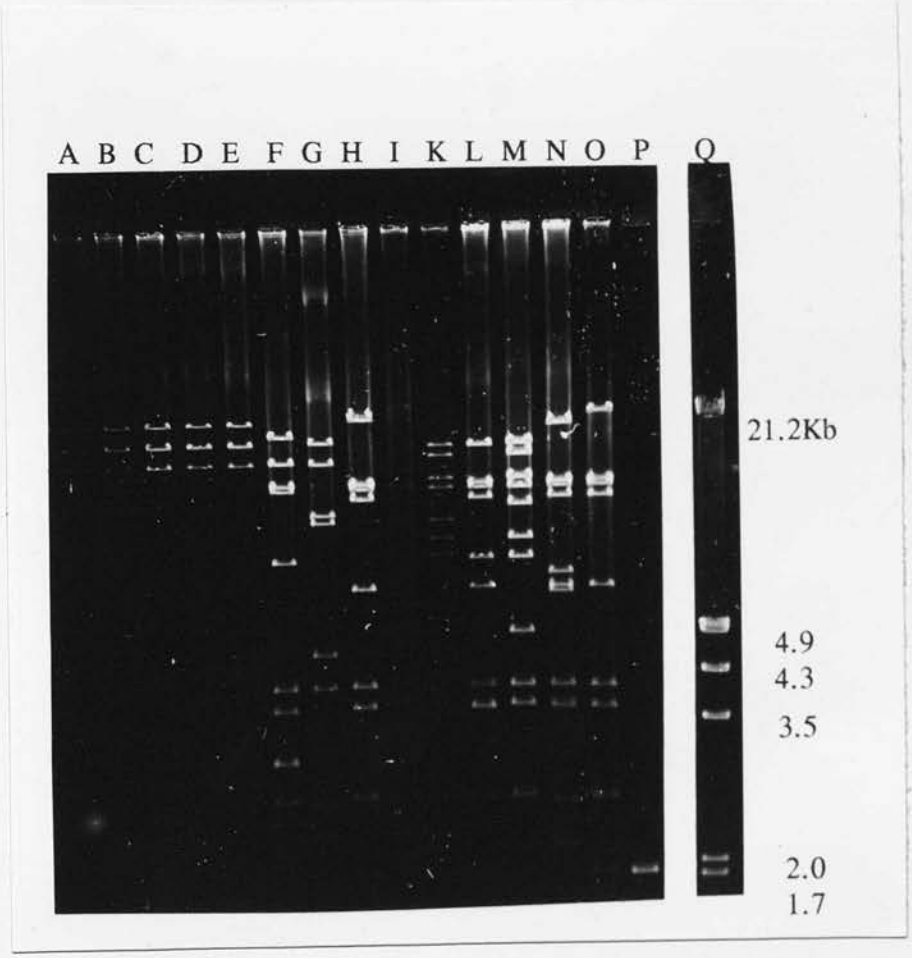


Figure 24. Southern blot of *Cla*1 restriction endonuclease digests of plasmids harbouring the *dhfr*IV gene from this study (1989) and the study by Young *et al* (1986a) (pUK1123-pUK1109), hybridised with the DNA probe for the *dhfr*IV gene.

Tracks are transconjugant plasmid DNA *Cla*1 (numbers in parentheses indicate original donor strain). (A) *dhfr*IV probe fragment; (B) pUK1123; (C) pUK1119; (D) pUK1118; (E) pUK1116; (F) pUK1115; (G) pUK1114; (H) pUK1109; (I) pUK2026 (179); (J) pUK2019 (118); (K) pUK2014(36); (L) pUK2013 (35); (M) pUK2012 (34); (N) pUK2007 (16); (O) pUK2005 (10).

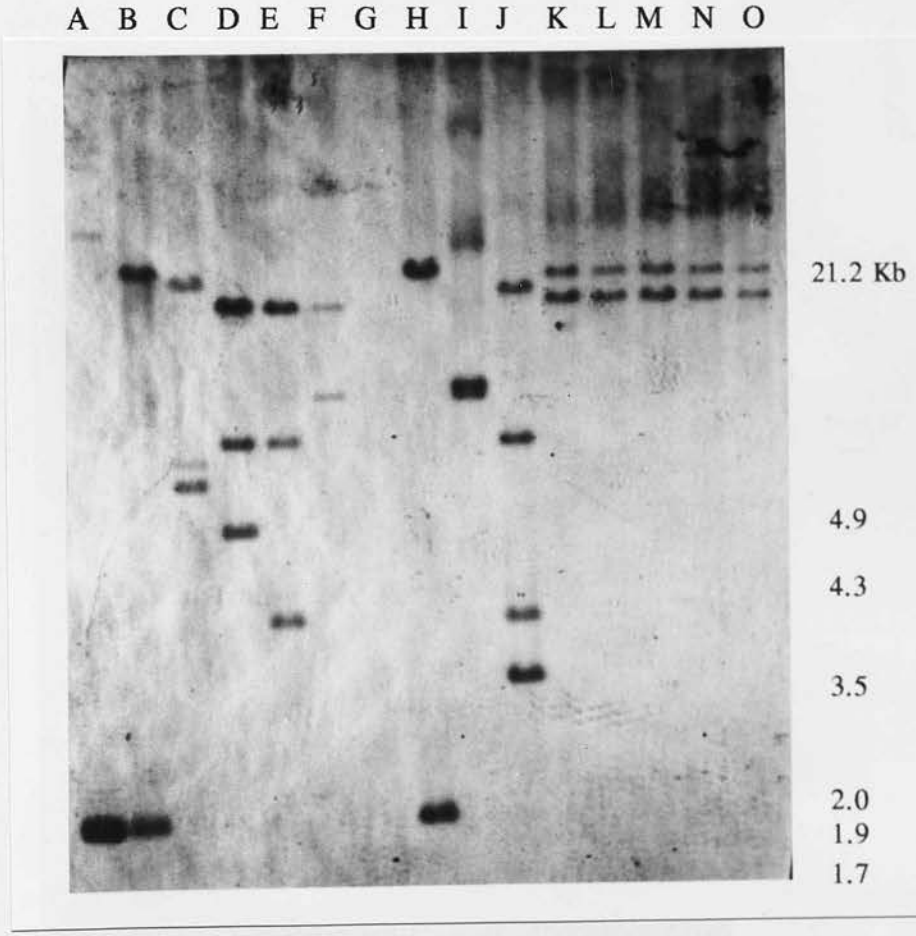


Figure 25. *Eco*R1 restriction endonuclease digests of plasmids harbouring the *dhfr*IV gene from this study (1989) and the study by Young *et al* (1986a) (pUK1123 pUK1114).

Tracks are transconjugant plasmid DNA digested with *Eco*R1, (numbers in parentheses indicate original donor strain). (A) pUK2005 (10); (B) pUK2007 (16); (C) pUK2012 (34); (D) pUK2013 (35); (E) pUK2014 (36); (F) pUK2019 (118); (G) pUK2026 (179); (H) pUK1123; (I) pUK1116; (J) pUK1119; (K) pUK1115; (L) pUK1109; (M) pUK1118; (N) pUK1114; (O) λ DNA *Hin*dIII digest marker.

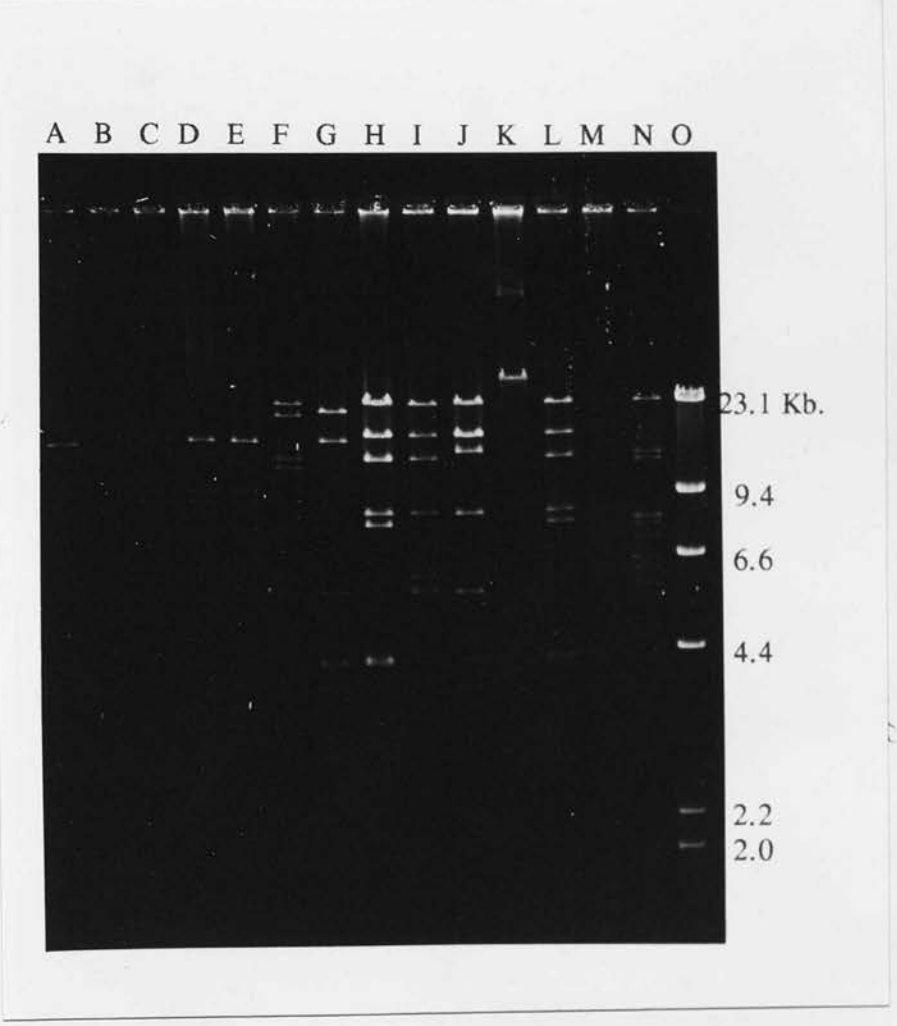
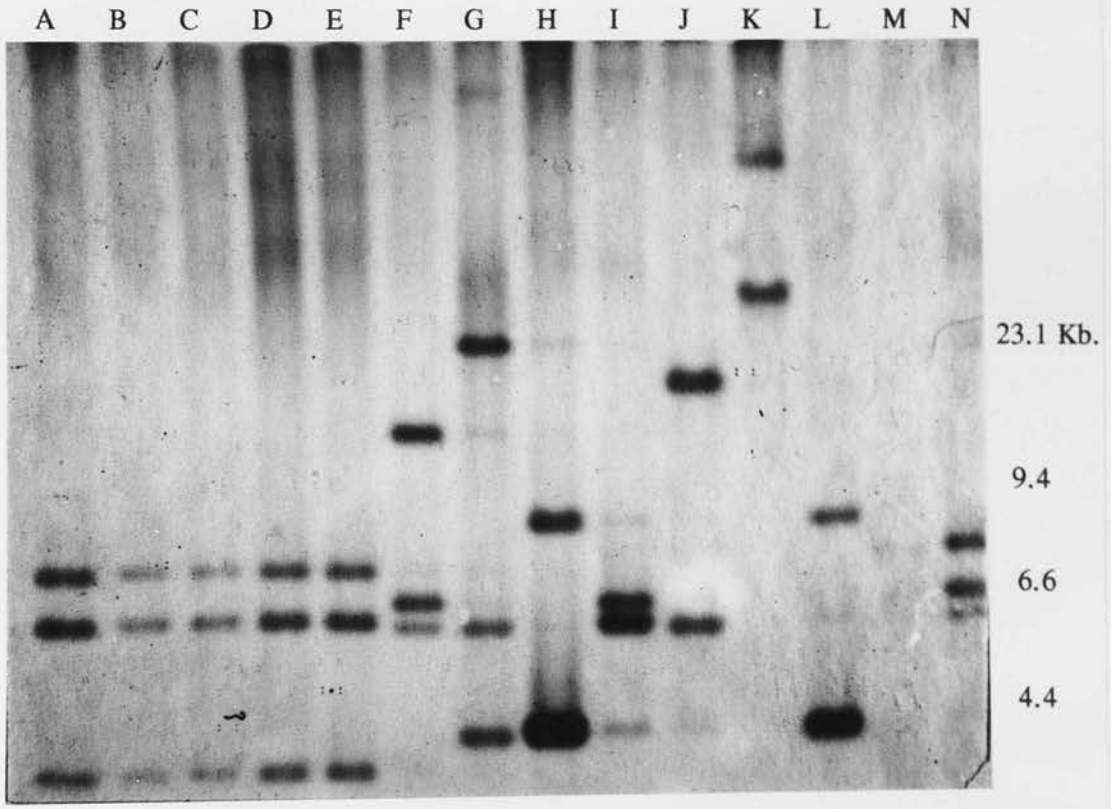


Figure 26. Southern blot of *Eco*R1 restriction endonuclease digests of plasmids harbouring the *dhfr*IV gene from this study (1989) and the study by Young *et al* (1986a) (pUK1123, pUK1114), hybridised with the DNA probe for the *dhfr*IV gene.

Tracks are transconjugant plasmid DNA digested with *Eco*R1 (numbers in parentheses indicate original donor strain). (A) pUK2005 (10); (B) pUK2007 (16); (C) pUK2012 (34); (D) pUK2013 (35); (E) pUK2014 (36); (F) pUK2019 (118); (G) pUK2026 (179); (H) pUK1123; (I) pUK1116; (J) pUK1119; (K) pUK1115; (L) pUK1109; (M) pUK1118; (N) pUK1114.



4.1.2. MICs on Different Media of *E. coli* J62-2 Carrying the *dhfr*IV Plasmids

The MICs of the plasmids carrying the type IV *dhfr*, in *E. coli* J62-2, were determined on different media. Thus a comparison could be made between the plasmids isolated in 1989 and those isolated in 1984, in which the MICs depended on the medium used for testing; the important factors affecting the MIC were shown to be methionine, glycine and adenine (Young and Amyes 1986a).

Table 24. MICs determined on different media for *E. coli* J62-2 carrying the type IV *dhfr* plasmids from this study and pUK1123 and pUK1114 from 1984

Plasmid	Donor strain	ISTA	DSTA	Minimal medium	MGA
pUK2005	10	2	8	16	4
pUK2007	16	2	8	16	4
pUK2012	34	2	8	16	4
pUK2013	35	2	8	16	4
pUK2014	36	2	8	16	4
pUK2019	118	16	64	64	32
pUK2026	179	16	64	256	256
1984					
pUK1123		4	8	16	4
pUK1114		128	64	128	128

ISTA is Isosensitest agar;

DSTA is diagnostic sensitest agar; minimal medium is Davis and Mingioli minimal salts containing glucose, proline, histidine and tryptophan;

MGA is this medium with the addition of methionine, glycine and adenine.

Again the plasmids carrying the type IV *dhfr* gave different MICs according to the medium used (table 24). Although the same degree of change could not be achieved as was shown by Young and Amyes (1986a), there was a marked increase in MIC when the assay was conducted on minimal medium as opposed to minimal medium containing methionine, glycine and adenine, or in complex medium.

4.1.3. Biochemistry of Two of the DHFR Type IV Enzymes Identified By the DNA Probe

One of the plasmids gave MICs that were significantly higher than those given by the other plasmids from the 1989 study or most of the plasmids from the 1984 study, except two that showed similarly raised MICs. The biochemical properties of the DHFRs of the plasmids pUK2007 and pUK2026 were investigated for comparison with those of the DHFR from plasmid pUK1123, the plasmid in which the DHFR type IV was originally characterized.

Table 25. Summary of properties of DHFRs from pUK2007, pUK2026 and pUK1123.

Plasmid	Tp ID_{50} μM	Mtx ID_{50} μM	TD_{50} min	DHF K_m μM	Tp K_i μM	Size Kd	MIC in ISTA mg/L
pUK1123	0.20	0.020	>12	37.0	0.063	46.7	4
pUK2007	0.14	0.002	>12	9.1	0.020	46.0	2
pUK2026	0.10	.012	>12	20.0	0.040	45.0	16

Tp ID_{50} is the concentration of trimethoprim required to give 50% inhibition.
Mtx ID_{50} is the concentration of methotrexate required to give 50% inhibition.
TD_{50} is the time in minutes of exposure of the enzyme to a temperature of 42°C required to reduce the activity by 50%.
DHF K_m is the Michaelis constant for DHF.
Tp K_i is the inhibition constant for trimethoprim against DHF.

The properties of the DHFRs carried by pUK2026 and pUK2007 were very similar to the original DHFR type IV carried by pUK1123 (table 25). There was some discrepancy between the methotrexate ID_{50} of the DHFR of pUK2007, pUK2026 and the original type IV. The methotrexate ID_{50} of pUK1123 and pUK2026 were much closer than pUK2026 and pUK2007. The other biochemical data, however, combined with the hybridisation results, indicated that these enzymes are very similar. Although pUK2026 had a higher MIC than pUK1123, the enzyme encoded by pUK2026 seemed to have very similar properties to the enzyme carried by pUK1123. Indeed it showed very slightly less resistance to trimethoprim, although this was probably experimental error.

4.2. INDUCTION OF THE DHFR TYPE IV

4.2.1. Induction of the DHFR Encoded by pUK2007

The DHFR of plasmid pUK2007 was very like the type IV DHFR of pUK1123. This enzyme has been shown to be inducible when challenged with trimethoprim. In order to determine if this mechanism has persisted in the plasmids isolated in 1989, induction of the DHFR encoded by pUK2007 was compared to that of plasmid pUK1123. Both plasmids were in *E. coli* J62-2 transconjugants (table 26).

Table 26. Induction of the DHFR of *E. coli* J62-2 (pUK2007) in comparison to that of *E. coli* J62-2 (pUK1123).

DHFR of plasmid	Trimethoprim (mg/L)	Specific activity (nmol DHF reduced/min/mg protein)
pUK1123	0	2.6
pUK1123	50	834.7
pUK1123	150	1047.4
pUK2007	0	3.8
pUK2007	50	362.2
pUK2007	150	578.2

The specific activity of both DHFRs increased dramatically in response to challenge with increasing concentrations of trimethoprim (table 26). Thus that unusual property of the DHFR type IV, of being inducible, has been maintained in the plasmid pUK2007.

This initial experiment was performed in transconjugants, to provide an isogenic background for the experiments. The following experiment was performed on the wild-type strain harbouring pUK2007, strain 16T, in order to determine if this system also operated in the wild-type, as isolated from the normal flora (table 27).

Table 27. Induction of the DHFR in the wild-type strain 16T.

Trimethoprim (mg/L)	Specific activity (nmol DHF reduced/min/mg protein)
0	10.0
40	62.0

Challenging the wild-type strain harbouring plasmid pUK2007 with trimethoprim produced higher levels of DHFR but to a lesser degree than was seen in the standard *E. coli* J62-2 (table 27). The wild-type strain had a higher MIC on IST agar, 32mg/L as compared to 4mg/L for the transconjugant, which may reflect decreased permeability to trimethoprim. This and other factors may influence the expression of the DHFR, thus all further experiments were carried out in the standard laboratory strain.

4.2.2. Expression of the DHFR of pUK2026 Compared to pUK1123

The *E. coli* J62-2 transconjugant carrying pUK2026 had a higher MIC than those carrying pUK1123 or pUK2007, although it had very similar properties, hybridised with the type IV probe and displayed the differential MICs when these were performed on different media. The expression of the DHFR from this plasmid was compared to that of the DHFR type IV of pUK1123 (table 28).

The DHFR of plasmid pUK2026 did not appear to be as inducible as the other DHFR type IVs of pUK1123 and pUK2007. It is produced constitutively at moderate levels compared to the expression of the DHFR of pUK1123 (table 28). This may account for the greater MIC, the constitutive production of moderate levels of DHFR type IV resulting in a higher MIC than that given by low level expression in the plasmids carrying the inducible enzyme. This assumes that no induction takes place on the agar plate, a matter that will be considered later.

Table 28. Comparison of DHFR expression from pUK2026 and pUK1123.

Plasmid	Trimethoprim (mg/L)	Specific activity (nmol DHF reduced/min/mg protein)
pUK1123	0	0.8
pUK1123	40	675.0
pUK1123	160	1586.0
pUK2026	0	25.0
pUK2026	40	38.0
pUK2026	160	17.0

4.2.3. The Effect of Thymine Starvation on the Induction of the DHFR Type IV

Thomson *et al* (1993) demonstrated that the presence of thymidine, the ribonucleoside derivative of thymine, abolishes the induction of the type IV DHFR of pUK1123. Trimethoprim has the effect of producing thymine starvation in bacterial cells when methionine, glycine and a purine are present in the medium. The role of thymine may thus be central to the induction of the type IV DHFR. Therefore, in order to investigate whether thymine starvation was sufficient to produce induction of the DHFR type IV, a thymine requiring auxotroph of *E. coli* J62-2 was selected, and the plasmid pUK1123 was transferred into it.

To produce the auxotroph, *E. coli* J62-2 was grown in Davis and Mingioli minimal medium containing proline, histidine and tryptophan, together with trimethoprim at 40mg/L and thymidine at 50mg/L. Thymidine was used in preference to thymine because it is more readily taken up by the cell. This was cultured for five days to allow thymine requiring cells, which are resistant to trimethoprim, to grow through. From this culture, 0.1ml was spread on agar containing the same concentration of supplements. Colonies from this medium were tested for thymine auxotrophy and *E. coli* J62-2 auxotrophic markers.

Plasmid pUK1123 was transferred to this *E. coli* J62-2, confirmed as a thymine requiring auxotroph, from *E. coli* J53. Streptomycin at 20mg/L was used as the selective agent for plasmid transfer.

The resulting transconjugant, *E. coli* J62-2 *thy* (pUK1123), was then subjected to growth in minimal medium containing decreasing concentrations of thymidine, and the specific activity of the DHFR from the cell free extract measured (table 29).

When the thymine requiring strain was grown in reduced thymidine concentrations, an increase in DHFR specific activity of up to ten-fold could be seen between cells grown in 10mg/L and in 1mg/L thymidine (table 29). This effect is reversed in cells grown in of thymidine, 0.5mg/L; however, this may be as a result of the over-growth of *thy*$^+$ revertants in a medium which is very low in thymidine.

Table 29. DHFR specific activity of thymine requiring auxotroph in decreasing concentrations of thymidine.

Thymidine concentration (mg/L)	Specific activity (nmol DHF reduced/min/mg protein)
10	1.3
2	1.5
1	14.6
0.5	6.3

Thymine depletion produced an increase in the expression of DHFR which confirms the role of thymine in the induction mechanism of the type IV DHFR; thymine starvation was sufficient to induce greater expression of the DHFR type IV.

4.3. INDUCTION OF TEM-1 IN *E. COLI* J62 (pUK2007)

4.3.1. Induction of TEM-1 with Trimethoprim or Amoxycillin

The plasmid pUK2007 as well as harbouring the *dhfr*IV gene also carries the *bla* gene of the TEM-1 β-lactamase. It was speculated that the expression of this gene might also be affected in the same way as the expression of the *dhfr* IV gene. The expression of TEM-1 can be measured by spectrophotometry and the specific activity assayed. The MIC of ampicillin for *E. coli* J62-2 (pUK2007) is 256mg/L. This strain was challenged with varying concentrations of ampicillin and trimethoprim at 80mg/L. The overnight culture was then prepared for the assay of both DHFR and β-lactamase specific activity; TEM-1 activity was assayed by the use of the chromogenic β-lactam substrate, nitrocefin (table 30).

Table 30. Specific activity of DHFR and β-lactamase from *E. coli* J62-2 (pUK2007) when challenged with ampicillin and trimethoprim.

Antibiotic	Concentration (mg/L)	β-lactamase specific activity[1]	DHFR specific activity[2]
control	0	0.50	1.6
ampicillin	80	0.98	1.3
ampicillin	160	1.14	-
ampicillin	240	1.93	4.0
trimethoprim	80	15.8	244.5

[1] μmol nitrocefin hydrolysed/min/mg protein.

[2] nmol DHF reduced/min/mg protein.

There were indications of a rise in β-lactamase specific activity after challenge with high concentrations of ampicillin, an increase of almost four-fold could be seen when the cells are challenged with 240mg/L of ampicillin. More surprisingly, however, is the rise in TEM-1 specific activity after challenge with trimethoprim, a very significant rise of 30-fold was recorded when the cells were challenged with 80mg/L of trimethoprim. This compared with an approximate increase of 150-fold in the DHFR specific activity (table 30).

4.3.2. Induction of the TEM-1 β-Lactamase and the DHFR Type IV with High Concentrations of Amoxycillin or Trimethoprim

From the previous experiment it was found that trimethoprim concentrations very much higher than the MIC, as derived on IST agar by the agar dilution method, are required to induce the DHFR type IV and the TEM-1 β-lactamase. Very high concentrations of the β-lactam may then be required to produce the induction of the TEM-1 enzyme. Therefore, the specific activity of the TEM-1 β-lactamase and the DHFR type IV from *E. coli* J62-2 (pUK2007) was assessed after challenge with trimethoprim at 40mg/L and amoxycillin at 500mg/L and 1000mg/L (table 31).

Table 31. Specific activity of the TEM-1 and DHFR type IV of *E. coli* J62-2 (pUK2007) after challenge with trimethoprim and high concentrations of amoxycillin.

Antibiotic	Concentration (mg/L)	DHFR specific activity[1]	β-lactamase specific activity[2]
control	0	9.9	0.01
trimethoprim	40	238.4	0.81
amoxycillin	500	6.6	0.34
amoxycillin	1000	37.3	1.46

[1]nmol DHF reduced/min/mg protein.

[2]μmol nitrocefin hydrolysed/min/mg protein.

Challenge with a high concentration of amoxycillin was capable of inducing increased specific activity in both the TEM-1 β-lactamase and the DHFR type IV (table 31). As in the case of induction with trimethoprim, induction with amoxycillin required the presence of levels of drug higher than those given by MICs derived by the agar dilution method. The observed increase in specific activity of the enzymes with the two drugs was not proportional, i.e. the rise in activity of each enzyme was not the same; for instance, challenge with amoxycillin at 500mg/L increases the specific activity of the TEM-1 β-lactamase but not that of the DHFR, and the observed rise in specific activity after challenge with trimethoprim was significantly higher in the case of TEM-1.

4.3.3. Induction of the TEM-1 β-Lactamase and the Type IV DHFR by Amoxycillin/Clavulanic Acid

The MIC of amoxycillin/clavulanic acid for *E. coli* J62-2 (pUK2007) was 8mg/L. The inhibition of TEM-1 by clavulanic acid, which is a competitive inhibitor of TEM-1, allows amoxycillin to take effect on the cell. Thus, considerably reduced concentrations of amoxycillin are required to produce cell death in the presence of clavulanic acid. The ability of amoxycillin in combination with clavulanic acid to produce induction of the TEM-1 β-lactamase and the DHFR type IV enzymes was tested (table 32).

Table 32. Specific activity of TEM-1 β-lactamase and DHFR type IV after challenge with amoxycillin/clavulanic acid.

Antibiotic	Concentration (mg/L)	DHFR specific activity[1]	β-lactamase specific activity[2]
control	0	0.29	0.33
trimethoprim	40	24.44	5.5
amox/clav*	8	5.35	1.06
amox/clav*	12	16.04	3.74

*Amoxycillin:clavulanic acid was used in a 2:1 ratio.

[1]nmol DHF reduced/min/mg protein.

[2]μmol nitrocefin hydrolysed/min/mg protein.

Much lower concentrations of the amoxycillin/clavulanic acid combination were capable of producing the induction of both enzymes (table 32). Significant increases in the specific activity of both enzymes were seen at amoxycillin/clavulanic acid concentrations around the MIC, as derived by agar dilution.

4.3.4. The Over-Expression of TEM-1 Is Reversible

Young *et al* (1993) demonstrated that the DHFR type IV in *E. coli* J62-2 (pUK1123) was induced by challenge with trimethoprim and that this was a reversible condition and not the result of the selection of an over-expressing mutant. Levels of DHFR type IV returned to basal levels when cells that had been

over-producing the enzyme in broth containing trimethoprim were sub-cultured into broth containing no drug.

To determine if this were true for the induction of the TEM-1 β-lactamase, a similar experiment was performed on *E. coli* J62-2 (pUK2007). Cells were challenged with of amoxycillin/clavulanic acid, 8mg/L, in a total volume of 200ml of IST broth; a control containing no drug was also included. After overnight culture, 1ml of the culture was added to fresh IST broth, containing no drug; the remainder was prepared for β-lactamase and DHFR specific activity assays. The fresh culture containing no drug was again incubated overnight with vigorous shaking as in all other assays, after which this was also prepared for β-lactamase and DHFR specific activity assays (table 33).

Table 33. β-lactamase and DHFR specific activities after challenge with amoxycillin/clavulanic acid and after subsequent sub-culture into broth containing no drug.

Concentration of amoxycillin/ clavulanic acid (mg/L)	Specific activities after challenge		Specific activities after sub-culture	
	DHFR[1]	β-lactamase[2]	DHFR[1]	β-lactamase[2]
0	1.01	0.02	5.8	0.16
amox/clav 8	12.2	0.5	3.4	0.18

[1]nmol DHF reduced/min/mg protein.

[2] μmol nitrocefin hydrolysed/min/mg protein.

Although the specific activities were variable, it can be seen that the levels of enzyme activity of both the DHFR and the β-lactamase returned to basal levels in the equivalent cultures containing no drug challenge after they have achieved activities 10-fold greater (table 33). This would indicate that the over-expression is not by the selection of mutants. In support of this, it would also seem unlikely that an unrelated antimicrobial such as trimethoprim could select for mutants resistant to β-lactam agents, by the over-expression of a β-lactamase, and vice-versa.

4.4. THE EFFECT OF INDUCTION ON THE MIC

4.4.1. Increase in MIC After Challenge with Antimicrobials

The MICs given on IST agar are significantly lower than the concentrations in which induction takes place and in which the cells will subsequently grow, at least in the case of trimethoprim and amoxycillin as a single agent. This would indicate that the enzymes are not induced when the cells are grown on agar. To investigate the possible affect of induction on the MIC, the test organism *E. coli* J62-2 (pUK2007) was incubated overnight with vigorous shaking in different concentrations of antimicrobials, in order to produce induction of the enzymes before the MIC assays were performed. The MICs were then determined by the normal agar dilution method. *E. coli* J62-2 (R1), which also carries TEM-1 was included as a control (table 34).

After challenge with antimicrobial, at a sufficiently high concentration of drug to produce induction of the enzymes, the MICs of trimethoprim, amoxycillin and amoxycillin/clavulanic acid rose substantially. A very large increase in the MIC of trimethoprim was seen, the cells being rendered resistant to what would be considered high levels of trimethoprim by an initial challenge with both trimethoprim and amoxycillin/clavulanic acid. No equivalent rise was seen in the MICs of amoxycillin or amoxycillin/clavulanic acid in the case of the R1 carrying strain (table 34).

The challenge by amoxycillin did not appear to be sufficient to produce the very large increase in MIC displayed when the cells were challenged with trimethoprim or the combination of amoxycillin and clavulanic acid. Thus, it can be seen that the cells do not induce the resistance enzymes on agar plates under normal circumstances. However, if the cells are challenged prior to assay, the induced enzymes allow the cells to grow in greatly increased concentrations of antimicrobial. As might be expected from the induction studies, challenge by one antimicrobial results in increased resistance to both types of agent, i.e. challenge by β-lactam resulted in increased resistance to both the β-lactam agents and trimethoprim, and challenge with trimethoprim resulted in increased resistance to both trimethoprim and the β-lactam agents.

Table 34. The MICs of trimethoprim, amoxycillin and amoxycillin/clavulanic acid determined after challenge with antimicrobials.

Plasmid	Drug concentration (mg/L) of challenge before assay	Trimethoprim MIC (mg/L)	Amoxycillin MIC (mg/L)	Amox/Clav* MIC (mg/L)
R1	0	-	512	8
R1	amoxycillin 250	-	512	8
R1	amoxycillin 500	-	512	8
R1	amoxycillin 1000	-	512	8
R1	amox/clav 4*	-	512	8
R1	amox/clav 8*	-	512	8
pUK2007	0	4	512	8
pUK2007	trimethoprim 40	128	1024	16
pUK2007	trimethoprim 80	>128	>1024	16
pUK2007	trimethoprim 160	>128	>1024	16
pUK2007	amoxycillin 250	4	512	8
pUK2007	amoxycillin 500	4	512	8
pUK2007	amoxycillin 1000	16	1024	16
pUK2007	amox/clav 4*	4	512	8
pUK2007	amox/clav 8*	128	1024	16
pUK2007	amox/clav 12*	128	1024	16

*Amoxycillin:clavulanic acid was used in a 2:1 rato.

4.4.2. MICS by Tube Assay for *E. coli* J62-2 (pUK2007)

MICs were assayed in 5ml of IST broth by overnight incubation at 37°C with vigorous shaking, the last tube showing no growth was taken as the MIC (table 35).

Table 35. MICs as determined by broth "tube" assay.

Plasmid	Amoxycillin MIC (mg/L)	Amox/clav* MIC (mg/L)	Trimethoprim MIC (mg/L)
pUK2007	>2048	512	64
R1	2048	16	-

*Amoxycillin:clavulanic acid was used in a 2:1 ratio.

Again, under conditions when the enzymes can be induced, the MICs were raised substantially (table 35). This was shown to greatest effect in the case of amoxycillin/clavulanic acid where the MIC was 64mg/L, making the cells resistant to amoxycillin/clavulanic acid, whilst the strain carrying the R1 plasmid had an MIC of 16mg/L, as determined by this method, whereas if assayed by agar dilution both strains had an MIC of 8mg/L.

4.5. THE EFFECT OF GROWTH PHASE AND CELL NUMBERS ON INDUCTION

4.5.1. The Affect of Growth-Phase on the Induction by Trimethoprim and Amoxycillin/Clavulanic Acid of *E. coli* J62-2 (pUK2007)

E. coli J62-2 (pUK2007) was grown for two hours, to approximately mid-log phase, and then challenged with amoxycillin/clavulanic acid (12, 32 or 64mg/L) or trimethoprim (40 and 512mg/L). The cells were harvested after two and 24 hours and assayed for TEM-1 β-lactamase or the DHFR type IV specific activity (table 36).

Table 36. Specific activities of TEM-1 and DHFR type IV after challenge of log-phase cells with various concentrations of drug after two or 24 hours.

Drug concentration (mg/L)	Two hours post-challenge		24 hours post-challenge	
	DHFR activity[1]	β-lactamase activity[2]	DHFR activity[1]	β-lactamase activity[2]
0	0.54	0.04	0.61	0.04
amox/clav 12	0.08	0.002	1.36	0.01
amox/clav 32	0.001	0.001	-	-
amox/clav 64	-	-	-	-
trimethoprim 40	0.19	0.07	0.64	0.02
trimethoprim 512	0.06	0.06	1.2	0.05

[1]nmol DHF reduced/min/mg protein.

[2]μmol nitrocefin hydrolysed/min/mg protein.

No induction of either the TEM-1 β-lactamase or the DHFR type IV was seen under these conditions. At high concentrations of amoxycillin/clavulanic acid, i.e. 32 or 64mg/L, the cells were killed. Gram-stains of these cultures after two hours showed lysed cells, few were intact, in the culture containing 32 mg/L, while the culture containing 64mg/L showed only cell debris after two hours. After 24 hours both showed only cell debris. These cells seem, however, to be able to overcome trimethoprim when high cell densities, in log-phase are challenged. A similar result was achieved in *E. coli* J62-2 (pUK1123) with trimethoprim as the challenge (results not shown).

4.5.2. The Effect of Cell-Phase on the Induction Mechanism

Experiments were performed to separate the effect of cell numbers and the effect of cell phase on the induction mechanism of the type IV DHFR. A culture of *E. coli* J62-2 (pUK2007) was grown overnight in IST broth. Of this culture 0.1ml was diluted in 10ml of IST broth and incubated with vigorous agitation for two hours to approximately mid-log phase. The remainder of the culture was maintained at 37°C. Of the two hour mid-log phase culture, one ml was treated with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) to a final concentration of 5mM. (This is an uncoupler of the electron motive force, and thus inhibits cell metabolism).

The treated cells were incubated on ice for five minutes; they were then washed in Davis and Mingioli medium before being resuspended in one ml of IST broth.

The overnight culture was diluted to approximately the same cell density as the mid-log phase cells, as measured at O.D. 540nm. IST broths containing trimethoprim with 0 or 40mg/L, were then inoculated with 0.1ml of each of the three cell suspensions. The cell numbers at inoculation and after two hours were checked by performing viable counts (table 37). The broths were cultured overnight and DHFR assays performed on the cell extracts (table 38).

Table 37. Cell counts of overnight, carbonyl cyanide *m*-chlorophenyl hydrazone treated and log phase inocula.

Cell suspension	Inoculum (cfu/ml)	Cell concentration after two hours (cfu/ml)	
		trimethoprim 0 mg/L	trimethoprim 40 mg/L
overnight	$3x10^3$	$6x10^7$	$7x10^3$
CCCP	$2x10^3$	$6x10^7$	$2x10^4$
log phase	$8x10^3$	$8x10^7$	$4x10^4$

The overnight culture, which was presumably in stationary phase, induced high levels of both the TEM-1 β-lactamase and the DHFR. Those cells that had their metabolism disrupted, and were forced back into stationary phase by the action of CCCP, also produced a considerable increase of both enzymes. However, challenge of cells during mid-log phase resulted in a very much reduced induction of the DHFR type IV and showed a decrease in the production of the TEM-1 enzyme (table 38).

If the figures were expressed in terms of relative increase in DHFR type IV and TEM-1 β-lactamase specific activity between challenged and unchallenged cultures in each of the three inoculum types, the low level of induction in log phase cells could be seen. In the overnight cells the increase was 1458-fold in the DHFR, six-fold in the β-lactamase; in the CCCP treated cells induction was less marked being some 20-fold in the DHFR and four-fold in the β-lactamase; in the log phase

cells there was only a three-fold increase in DHFR activity and a reduction in β-lactamase activity.

The cell numbers in the inocula were similar (table 37), thus inoculum size could not be affecting the induction process. The cells treated with CCCP seemed to recover after two hours when cell numbers were similar to the other cultures; the log-phase cells grew slightly more rapidly than the cells inoculated at stationary phase.

Table 38. DHFR specific activities of cultures inoculated with cells in different phases.

Cells in inoculum	Trimethoprim challenge (mg/L)	DHFR specific activity[1]	β-lactamase specific activity[2]
overnight	0	0.12	0.07
overnight	40	175.00	0.45
CCCP	0	1.4	0.11
CCCP	40	28.6	0.46
log phase	0	4.06	0.14
log phase	40	12.52	0.06

[1]nmol DHF reduced/min/mg protein

[2]μmol nitrocefin hydrolysed/min/mg protein

The CCCP, although not a complete inhibitor of metabolism was sufficient to reduce the metabolic processes so that the cells behaved like stationary phase cells. The enzymes were induced in these cells on treatment with trimethoprim, although not to the same extent as the overnight culture. This presumably resulted from the incomplete action of the CCCP treatment on the cells. The cells challenged during log-phase showed little induction of the DHFR type IV, and none of the TEM-1 β-lactamase. Thus, cell phase seems to be very important in the induction mechanism; induction did not take place in log-phase cells.

4.5.3. The Effect of Inoculum Size on the Induction Mechanism

Cell numbers in the inoculum may also have an effect on the induction process. In order to investigate the effect of inoculum size, 100ml of IST broth was inoculated

with *E. coli* J62-2 (pUK2007) and cultured overnight. The cells were harvested and resuspended in 10ml of Davis and Mingioli medium. Aliquots of 5, 0.5 and 0.05ml of this cell suspension were used to inoculate IST broth containing trimethoprim, 40mg/L; 0.05ml was also inoculated into IST broth containing no trimethoprim. Viable counts were performed on these cultures, and after overnight, growth cell free extracts were assayed for enzyme activity (table 39).

Table 39. Enzyme activity in cultures inoculated with differing inoculum sizes after challenge with trimethoprim.

Inoculum	Initial cell numbers (cfu/ml)	DHFR specific activity[1]	β-lactamase specific activity[2]
0.05	$4.6x10^6$	26.7	1.04
0.5	$5.2x10^7$	12.8	0.13
5.0	$5.4x10^8$	1.3	0.07
0.05 control no trimethoprim	-	3.3	0.18

[1]nmol DHF reduced/min/mg protein.

[2]μmol nitrocefin hydrolysed/min/mg protein.

Cell numbers clearly had an effect on the induction of the DHFR and TEM-1. When a large inoculum was used ($5.4x10^8$cfu/ml), induction of the DHFR and β-lactamase was abolished, whilst inoculation with moderate cell numbers ($5.2x10^7$cfu/ml) resulted in reduced induction (table 39). Thus both cell numbers and the growth phase of the cells when challenged with drug influence the induction of the type IV DHFR and TEM-1 β-lactamase.

4.5.4. DHFR Specific Activity and Cell-Phase

Samples of an untreated culture were taken at four hour intervals and assayed for DHFR specific activity (table 40).

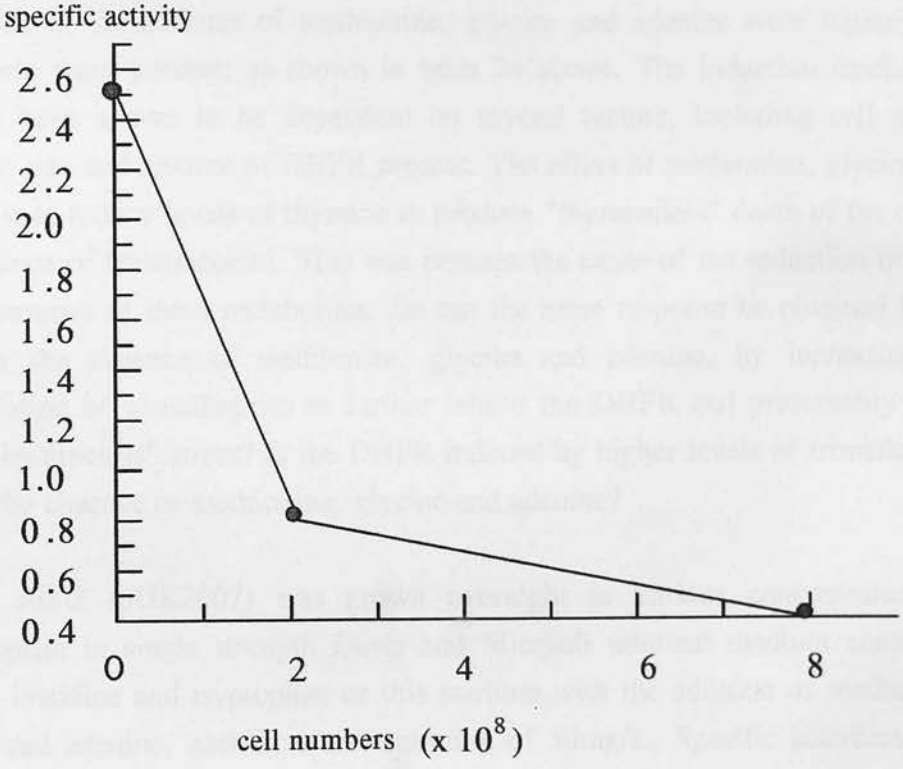
The specific activity of the DHFR dropped with time of culture (table 40 and figure 27). Unfortunately sufficient material could not be obtained to allow assay before four hours. At four hours, however, there was more activity than at eight or twelve hours when activity is reduced six-fold. In mid-log phase the cells appeared to produce more DHFR. This may have contributed to the reduction of the response in log-phase cells to treatment with trimethoprim, resulting in the abolition of the induction of DHFR. However, even at high concentrations of trimethoprim no induction was observed; thus other factors may be involved in the response of the cells in log-phase.

Table 40. The correlation of DHFR specific activity with cell growth.

Time (hours)	cell numbers (cfu/ml)	DHFR specific activity*
4	2.9×10^6	2.5
8	2.1×10^8	0.8
12	8.0×10^8	0.4

*nmol DHF reduced/min/mg protein

Figure 27. Graph of increasing cell numbers with time against DHFR specific activity (nmol DHF reduced/min/mg protein), as given from the data above.



4.6. THE EFFECT OF THE METABOLITES METHIONINE, GLYCINE, ADENINE AND THYMINE

4.6.1. Induction of the Type IV DHFR in the Presence and Absence of Methionine, Glycine and Adenine

The MICs in the absence of methionine, glycine and adenine were higher than when these were present, as shown in table 24 above. The induction mechanism has also been shown to be dependent on several factors, including cell phase, inoculum size and amount of DHFR present. The effect of methionine, glycine and adenine is to reduce levels of thymine to produce "thymineless" death of the cell in the presence of trimethoprim. This was perhaps the cause of the reduction of MIC in the presence of these metabolites. So can the same response be obtained in the cells, in the absence of methionine, glycine and adenine, by increasing the concentration of trimethoprim to further inhibit the DHFR and presumably bring about "thymineless" stress? Is the DHFR induced by higher levels of trimethoprim despite the absence of methionine, glycine and adenine?

E. coli J62-2 (pUK2007) was grown overnight in various concentrations of trimethoprim in single strength Davis and Mingioli minimal medium containing proline, histidine and tryptophan or this medium with the addition of methionine, glycine and adenine, each at a concentration of 50mg/L. Specific activities were then determined (table 41).

In order to compare this set of results they can be expressed more easily in terms of the relative increase in specific activity between the unchallenged and those challenged with trimethoprim in each case (table 42).

In media containing methionine, glycine and adenine, less trimethoprim was required to bring about induction of both the DHFR type IV and TEM-1 β -lactamase, although the reaction of the β-lactamase was not as pronounced (table 42). Even with trimethoprim at a concentration of 240mg/L, the induction of the DHFR was not as great in media lacking the metabolites as it was at a trimethoprim concentration of 40mg/L in media containing methionine, glycine and adenine.

Table 41. Specific activities of cells challenged with trimethoprim in the presence or absence of methionine, glycine and adenine.

Medium	Trimethoprim (mg/L)	DHFR specific activity[1]	β-lactamase specific activity[2]
PHT	0	0.88	0.09
PHT	40	1.40	0.17
PHT	120	4.40	0.52
PHT	240	5.56	1.26
MGA	0	0.30	0.09
MGA	40	3.48	0.39

PHT is Davis Mingioli media containing proline, histidine and tryptophan.
MGA is this media with methionine, glycine and adenine.
[1]nmol DHF reduced/min/mg protein.
[2]μmol nitrocefin hydrolysed/min/mg protein.

Table 42. Relative increase in specific activity between challenged and unchallenged cultures.

Medium	Trimethoprim (mg/L)	Relative increase in DHFR specific activity.	Relative increase in β-lactamase specific activity.
PHT	0	1	1
PHT	40	1.6	1.7
PHT	120	5.0	5.8
PHT	240	6.3	14
MGA	0	1	1
MGA	40	11.6	4.3

PHT is Davis Mingioli media containing proline, histidine and tryptophan.
MGA is this media with methionine, glycine and adenine.

4.6.2. The Effect of Methioinine, Glycine and Adenine on the Expression of the TEM-1 β-Lactamase *E. coli* J62-2 (pUK2007)

The metabolites, methionine, glycine and adenine, influence the expression of the type IV DHFR. Will the presence of these metabolites have the same influence on

the expression of the TEM-1 β-lactamase and will they have any influence on the ability of amoxycillin/clavulanic acid to produce induction of the resistance enzymes?

The MIC for the combination of amoxycillin/clavulanic acid on media containing methionine, glycine and adenine and media lacking these metabolites, with and without challenge prior to performing the assay, was first determined (table 43).

Table 43a. The MIC of amoxycillin/clavulanic acid for *E. coli* J62-2 (pUK2007) in minimal medium and medium containing methionine, glycine and adenine, with and without prior challenge.

Drug and concentration of challenge (mg/L)	MIC in minimal media[1] (mg/L)	MIC in methionine, glycine and adenine (mg/L)[2]
0	4	4
trimethoprim 40	16	16
amox/clav 8	16	16
amox/clav 12	16	16

[1] This is Davis and Mingioli (DM) minimal medium containing proline, histidine and tryptophan.[2] DM medium containing the additives methionine, glycine and adenine.

There was no difference in the MICs of amoxycillin/clavulanic acid when the assays were carried out in the presence or absence of methionine, glycine and adenine. The MICs were again raised when the cells were challenged and the resistance enzymes induced prior to assay of the MIC (table 43a).

To investigate the effect of the presence of the metabolites on the expression of TEM-1 β-lactamase, cells were challenged with trimethoprim in the presence and absence of the three metabolites and the specific activity of the TEM-1 β-lactamase assayed. In a further experiment, cells were challenged with amoxycillin/clavulanic acid also in the presence and absence of methionine, glycine and adenine, and again the specific activity of the β-lactamase was assayed (table 43b).

Table 43b. β-lactamase specific activities after challenge with trimethoprim in the presence and absence of methionine, glycine and adenine. The relative increase in specific activity from unchallenged cells is also shown.

Media	Concentration of challenge with trimethoprim (mg/L)	β-lactamase specific activity[1]	Relative increase
PHT	0	0.09	1
PHT	40	0.17	1.9
PHT	120	0.52	5.8
PHT	240	1.26	14.0
MGA	0	0.09	1
MGA	40	0.39	4.3

PHT is Davis Mingioli medium containing proline, histidine and tryptophan.
MGA is this medium with methionine, glycine and adenine.
[1]μmol nitrocefin hydrolysed/min/mg protein.

β-lactamase expression reacted in the same manner as the DHFR when the cells were challenged with trimethoprim in the presence of methionine, glycine and adenine (table 43b). Significantly less drug was required to bring about a similar rise in specific activity of TEM-1 β-lactamase when methionine, glycine and adenine were present in the media.

This feature of trimethoprim action was not repeated in the case of challenge with the amoxycillin/clavulanic acid combination. A greater increase in enzyme specific activity, both β-lactamase and DHFR, was seen when the cells were challenged in the absence of methionine, glycine and adenine (table 44). This possibly resulted from the greater effect of the drug in a nutrient depleted medium. It would be expected that the action of the β-lactam would not be influenced by the presence of the metabolites since their action is on the metabolic pathway on which trimethoprim acts. Once again both resistance mechanisms responded in the same way. Both the β-lactamase and the DHFR specific activities were not influenced by the presence of methionine, glycine and adenine when the cells were challenged with amoxycillin/clavulanic acid.

Table 44. β-lactamase and DHFR specific activities after challenge with amoxycillin/clavulanic acid in the presence and absence of methionine, glycine and adenine. The relative increase in specific activity from unchallenged cells is also shown.

Media	Concentration of amox/clav (mg/L)	DHFR specific activity[1]	Relative increase	β-lactamase specific activity[2]	Relative increase
PHT	0	2.8	1	0.14	1
PHT	4	12.7	4.5	0.36	2.5
PHT	8	660.9	236.0	5.3	38.0
PHT	12	no growth	-	-	-
MGA	0	4.4	1	0.21	1
MGA	4	3.5	0.8	0.28	1.3
MGA	8	41.9	9.5	2.1	10

PHT is Davis Mingioli medium containing proline, histidine and tryptophan.
MGA is this medium with methionine, glycine and adenine.
[1]nmol DHF reduced/min/mg protein.
[2] mmol nitrocefin hydrolysed/min/mg protein.

4.6.3. The Effect of Thymine Starvation on the Induction of TEM-1 β-lactamase

The plasmid pUK2007 was transferred, by conjugation, into the thymine requiring mutant of *E. coli* J62-2, as was outlined previously in the transfer of plasmid pUK1123 to this same strain.

This strain was then subjected, as in the previous experiment, to growth conditions of decreasing concentrations of thymidine. The specific activities of both the TEM-1 β-lactamase and the type IV DHFR were then assayed (table 45).

Table 45. The specific activities of DHFR and TEM-1 in the thymine requiring strain of *E. coli* J62-2 (pUK2007).

Thymidine concentration (mg/L)	DHFR specific activity[1]	β-lactamase specific activity[2]
1	1.3	0.28
2	1.1	0.27
5	0.6	0.17
10	0.8	0.20
50	0.8	0.14

[1]nmol DHF reduced/min/mg protein.

[2]mmol nitrocefin hydrolysed/min/mg protein.

The specific activity dropped slightly for both the β-lactamase and the DHFR, although the amounts were not very large, when the amount of thymidine in the growth medium was increased (table 45). However, both enzymes react in a similar way; both fell when thymidine, 5mg/L, was present, which may lend some credibility to the result. Thus, thymine deficiency seems to influence the expression of both enzymes.

4.7. THE EFFECT OF AMOXYCILLIN/CLAVULANIC ACID AND TRIMETHOPRIM ON THE GROWTH AND MORPHOLOGY OF *E.COLI* J62-2 (PUK2007)

E. coli J62-2 (pUK1123) undergoes an initial phase of cell death after inoculation in IST broth containing trimethoprim. In this phase, numbers dropped and the cells became elongated, a change in morphology that has been associated with thymineless-death; the cells then recovered to grow normally (Thomson *et al* 1993). To test if this response was also demonstrated in *E. coli* J62-2 (pUK2007), and if amoxycillin/clavulanic acid had a similar effect, three 200ml flasks of IST broth containing either no drug, trimethoprim 40mg/L or amoxycillin/clavulanic acid 12mg/L, were inoculated with *E. coli* J62-2 (pUK2007). These were incubated at 37°C with vigorous shaking for 24 hours. Samples were taken at 4 hour intervals for viable count and gram-film (figures 28 and 29).

Both the cells treated with trimethoprim and amoxycillin/clavulanic acid underwent an initial period of cell-death; cell numbers dropped from the numbers inoculated into the media (figure 28). The cells treated with trimethoprim started to recover at about four to eight hours, when the cell numbers began to increase steadily. The cells treated with amoxycillin/clavulanic acid, however, continued to fall in number until about 12 hours when there was a dramatic increase in cell numbers. The cells presumably started to recover when the amount of enzyme was sufficient to overcome the antibiotic, thus allowing normal cell growth to continue (figure 28). The cells themselves underwent morphological changes. *E. coli* J62-2 (pUK2007) was a small rod at inoculum; after four hours without drug the cells were slightly larger, however, with drug treatment the cells were greatly elongated (figure 29). The trimethoprim treated cells were elongated; as demonstrated by Thomson *et al* (1993). Those cells treated with amoxycillin/clavulanic acid are also elongated but they also appeared to have a bulge in the middle of the cell, perhaps where the cells may have divided. This feature was not apparent in the trimethoprim treated cells. On recovery, after 24 hours, the cells returned to the normal morphology, as shown by the untreated cells, although some elongated cells were also seen (figure 29).

The cells reacted in a similar, but not identical, manner to treatment with trimethoprim or amoxycillin/clavulanic acid. Both drugs brought about cell-death and morphological changes, involving cell elongation, before induction of the resistance mechanisms.

Figure 28. Growth of *E. coli* J62-2 (pUK2007) after challenge with trimethoprim or amoxycillin/clavulanic acid.

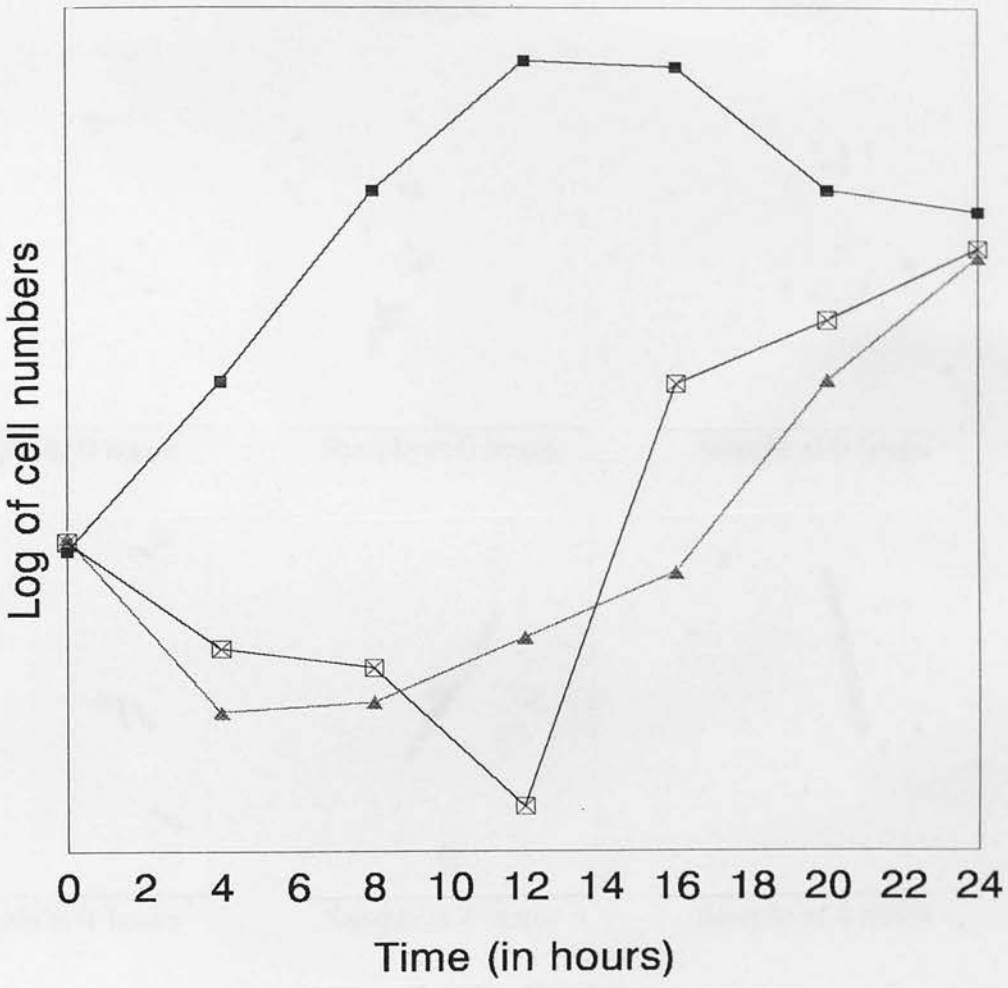
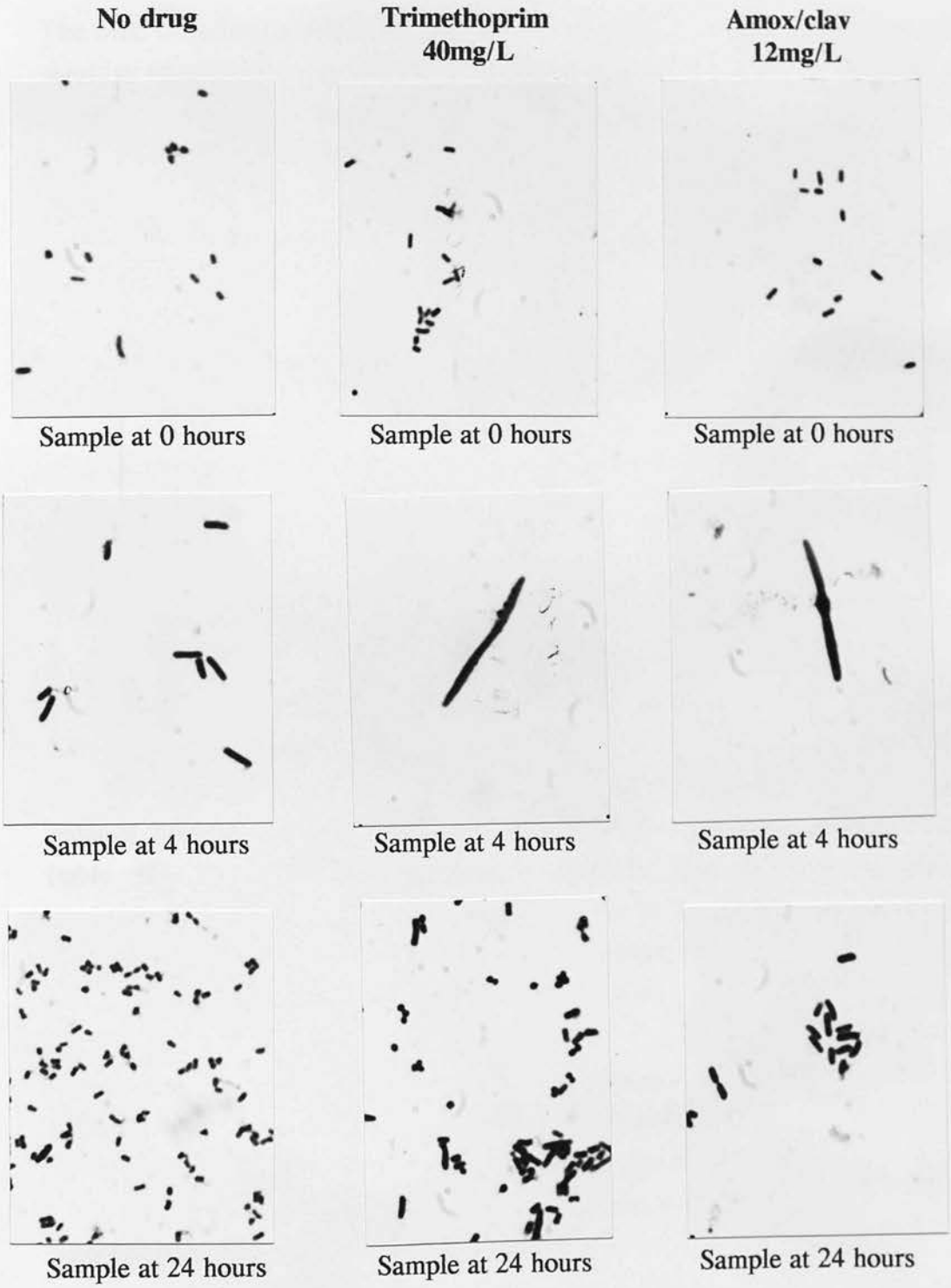


Figure 29. Photomicrographs of *E. coli* J62-2 (pUK2007) with (A) no drug (B) trimethoprim (40mg/L) and (C) amoxycillin/clavulanic acid (12mg/L)



4.8. DO OTHER ANTIBIOTICS PRODUCE INDUCTION OF THE DHFR TYPE IV AND TEM-1?

The MIC of nalidixic acid for *E. coli* J62-2 (pUK2007) is 8mg/L, the strain being sensitive to nalidixic acid. The MIC of streptomycin for this strain is 256mg/L, the resistance mechanism being located on the plasmid pUK2007. Induction was attempted with both of these agents.

Table 46. Specific activity of Type IV DHFRin *E. coli* J62-2 (pUK2007) after challenge with nalidixic acid.

Concentration of nalidixic acid (mg/L)	DHFR specific activity*
0	4.1
2	4.5
4	12.2
8	0.8
16	no growth

*nmol DHF reduced/min/mg protein.

There was an indication of increased specific activity when the cells were challenged with nalidixic acid at a concentration of 4mg/L. However, growth of the cells was inhibited by 8mg/L and the specific activity was affected by the much reduced growth of the cells. No growth was obtained with nalidixic acid at 16mg/L (table 46). The strain was sensitive to nalidixic acid and thus no possible mechanism could be induced to allow it to grow in increasing concentrations of nalidixic acid, as it could when treated with trimethoprim or amoxycillin. The cells perhaps died before any significant increase in DHFR could be achieved.

The plasmid pUK2007 carried a streptomycin resistance gene conferring an MIC of 256mg/L on *E. coli* J62-2. Is streptomycin capable of inducing the plasmid mediated resistance mechanisms? To test this, *E. coli* J62-2 (pUK2007) was challenged with streptomycin at concentrations of 256 and 512mg/L, and trimethoprim at 40mg/L, as a control (table 47).

Table 47. Specific activities of DHFR type IV and TEM-1 in *E. coli* J62-2 (pUK2007) after challenge with streptomycin.

Challenge (mg/L)	DHFR specific activity[1]	β-lactamase specific activity[2]
0	1.00	0.05
streptomycin 256	0.02	0.04
streptomycin 512	0.20	0.09
trimethoprim 40	82.90	0.13

[1]nmol DHF reduced/min/mg protein.

[2] μmol nitrocefin hydrolysed/min/mg protein.

Challenge with streptomycin produced no increase in specific activity of either the DHFR type IV or the TEM-1 β-lactamase (table 47). Thus, of the agents tested only trimethoprim, amoxycillin and amoxycillin/clavulanic acid were capable of inducing high levels of the resistance enzymes.

4.9. THE *bla* GENE OF TEM-1 AND THE *dhfr*IV GENE ARE NOT CLOSELY ASSOCIATED ON PLASMID pUK2007

The probe for the *dhfr* IV gives multiple bands; therefore the use of this probe and a probe for the TEM-1 *bla* gene could not be used to establish whether the two genes are linked. To try and demonstrate an association, a number of recombinants were created from different restriction digests of pUK2007 by selecting for recombinants with trimethoprim or ampicillin, and then checking for cross-resistance (table 48).

Of the six different recombinants formed from digests using "six-cutter" endonuclease restriction enzymes, no recombinant was formed that was resistant to both trimethoprim and ampicillin. It seemed likely, therefore, that they were not closely associated on the plasmid; if they had been it might be expected that they would have been inserted together during the cloning procedure. It seemed very unlikely indeed that the genes were transcribed from the same promoter. However, there remains the possibility that a mutation has occurred in one of the two genes forming a site for one of the above enzymes. Thus, the genes could have been cut by the enzyme.

Table 48. The following recombinants, formed from pUK2007, were created and characterized.

Plasmid	Enzyme	Vector	Approximate size of insert (Kb)	Resistance pattern of insert only
pUK2047	*Hin*dIII	pSU18	5	TpR, ApS
pUK2048	*Hin*dII	pSU18	4	TpS, ApR
pUK2049	*Pst*I	pSU18	4	TpR, ApS
pUK2052	*Eco*RI	pSU18	6	TpS, ApR
pUK2053	*Sal*I	pSU18	2	TpS, ApR
pUK2054	*Ava*I	pSU18	3	TpS, ApR

The 5Kb fragment of pUK2047, after separation and labelling with biotin, showed positive hybridisation with the 4Kb ampicillin resistance fragment of pUK2048. This would indicate the existence of repeated sequences associated with both the *dhfr*IV gene and the *bla* gene of TEM-1. These repeated sequences may be associated with the control of the induction mechanism, however, this would require further investigation (figures 30 and 31).

Figure 30. A 1% agarose gel of recombinant plasmids digested with *Hin*dIII.

(A) pUK2049; (B) pUK2047; (C) pUK1216, recombinant plasmid containing the *dhfr*IV gene; (D) pSU18; (E) λ *Hin*dIIIdigest.

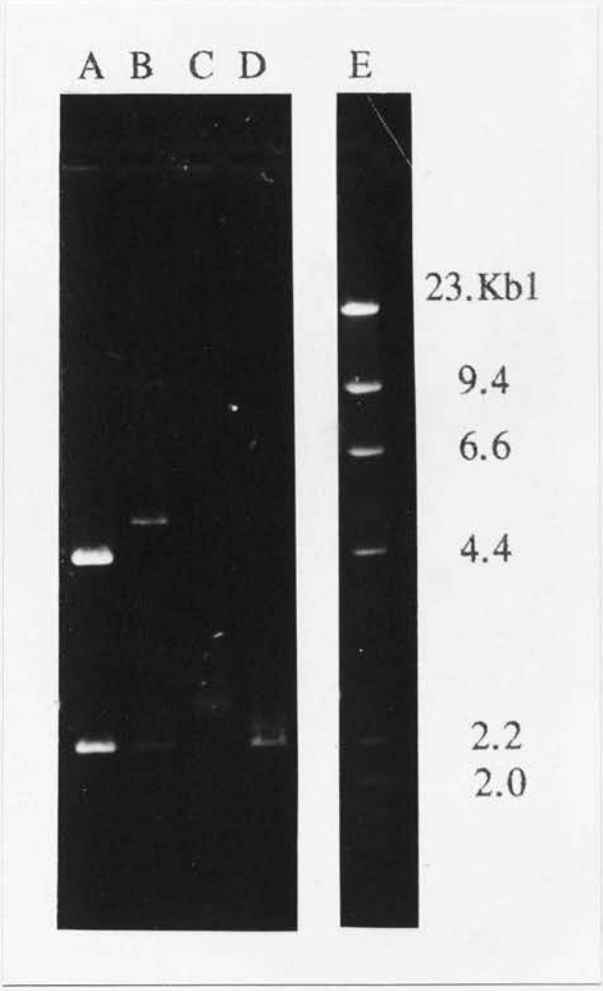
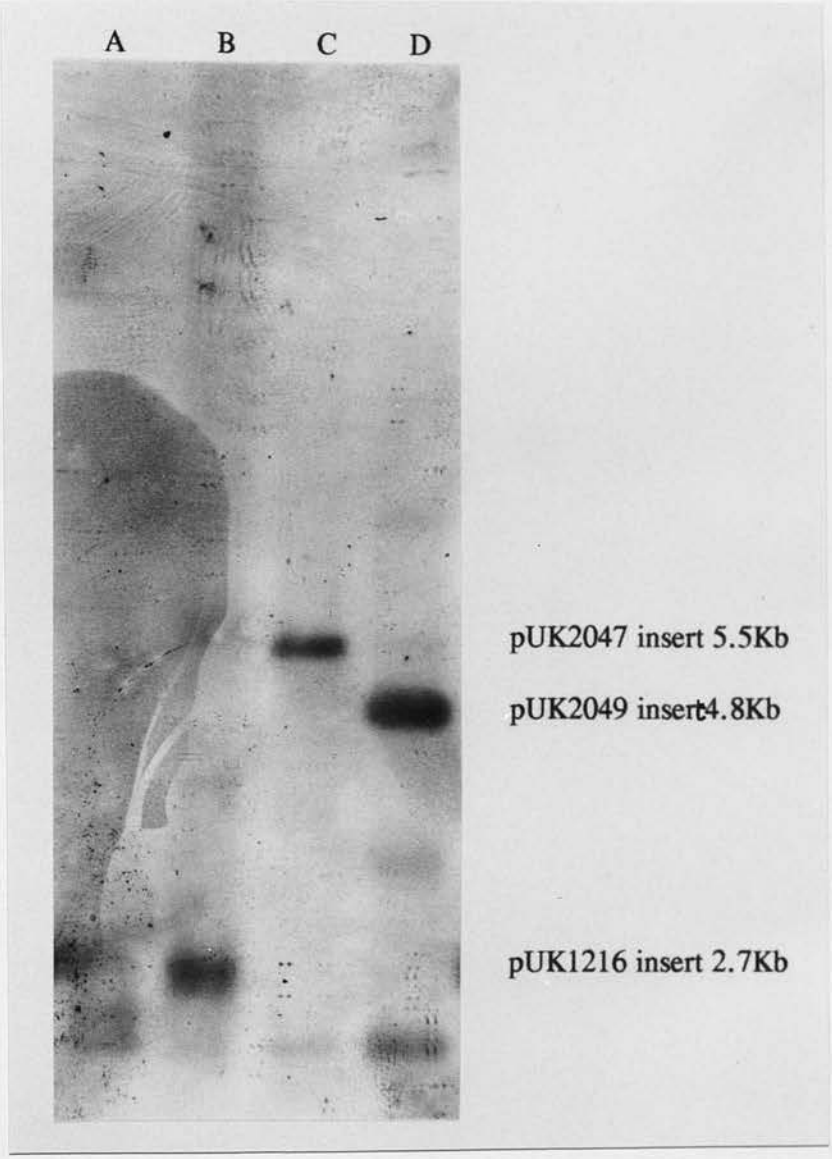


Figure 31. Southern blot of recombinant plasmids digested with *Hin*dIII and hybridised with the *dhfr*IV insert of pUK2049.

(A) pSU18; (B) pUK1216, recombinant plasmid containing the *dhfr*IV gene; (C) pUK2047; (D) pUK2049.



4.10. THE SPECIFIC ACTIVITIES OF RECOMBINANTS OF pSC101 CARRYING THE *dhfr*IV FROM pUK1123.

The following clones were created containing the *dhfr* IV gene inserted into the low copy number plasmid pSC101 (table 49). A low copy number vector was used in order that copy number of the recombinants would not influence expression of the DHFR.

Table 49. Low copy number recombinants carrying the *dhfr*IV gene.

Plasmid	Insert (Kb)	Derivation
pUK2043	6.5	sub-clone of pUK2041, a pUC18 recombinant containing an *Eco*R1 insert from pUK1123.
pUK2044	1.2	sub-clone of pUK1227, a pUC18 recombinant with a *Hin*dIII/*Hin*dII insert from pUK1123. A blunt-ended fragment from this recombinant was ligated with an *Eco*R1 linker and subsequently ligated with pSC101.

These recombinants were initially used to transform *E. coli* DH5α, from which plasmid DNA was purified and used to transform *E. coli* J62-2. The strains *E. coli* J62-2 (pUK2043) and *E. coli* J62-2 (pUK2044) were then used in induction experiments as before, being challenged with trimethoprim 50mg/L.

The recombinant plasmids were not capable of the induction response on challenge with trimethoprim (table 50). The DHFR was expressed at constitutive high levels, despite being on low copy number plasmids. Therefore, it may be that a repressor is present in the wild-type plasmid, that is not present in the recombinants, which suppresses the DHFR expression in the absence of trimethoprim.

The wild-type plasmid pUK1123, harbouring the original *dhfr*IV gene, was transfered into the strains harbouring the recombinant plasmid pUK2043. Transconjugants harbouring both plasmids were then selected and specific activities measured after challenge with trimethoprim (table 51).

Table 50. DHFR specific activities from recombinant plasmids in *E. coli* J62-2 after challenge with trimethoprim.

Plasmid	Trimethoprim (mg/L)	DHFR specific activity*
pUK1123	0	1.7
pUK1123	50	99.6
pUK2043	0	88.6
pUK2043	50	96.1
pUK2044	0	126.6
pUK2044	50	127.0

*nmol DHF reduced/min/mg protein.

Table 51. DHFR specific activities in *E. coli* J62-2 (pUK2043; pUK1123).

Plasmids	Trimethoprim (mg/L)	DHFR specific activity*
pUK2043	0	74
pUK2043	50	50
pUK1123; pUK2043	0	126
pUK1123; pUK2043	50	129
pUK1123; pUK2043	500	48
pUK1123; pUK2043	1000	25

*nmol DHF reduced/min/mg protein.

Again no induction was observed (table 51), thus no suppressor is present that will act in *trans* on the recombinant. It may be that this is a complex mechanism, as the results that have been presented would suggest, that requires large amounts of the plasmid DNA or is a property of the plasmid as a whole.

CHAPTER 5

THE NUCLEOTIDE SEQUENCE OF THE *dhfr*IV GENE

The nucleotide sequence of the *dhfr*IV structural gene was determined from pUK1227 (from Dr H-K Young) and an M13 sub-clone of the insert from pUK1227. The plasmid pUK1227 contained an insert from the original plasmid pUK1123 of approximately 1.2 Kb.

The nucleotide sequence of the *dhfr*IV gene was found to be 432 base pairs long, giving a translation product with a molecular weight of 17, 195. This is a similar size to the other plasmid mediated *dhfr* genes and their products, with the exception of the type IIs, and to the chromosomal DHFRs. A ribosomal binding site was identified which terminated 9bp upstream of the initiation codon ATG. A -10 promoter sequence was identified 12 bp upstream of this site, and a possible -35 promoter sequence was taken as being 17bp upstream of this sequence (labelled a). Both of these sequences show reduced homology with the *E. coli* consensus promoter sequences. Further possible -10 and -35 regions were identified (labelled b), with the -10 region starting 28bp upstream of the initiation codon, and the possible -35 sequence starting 20bp upstream of this region. This again is only a weak promoter in terms of the *E. coli* consensus promoter sequences.

A termination codon was identified which would give a protein of a size similar to the other DHFRs; however, no stem loop structure was seen associated with the termination of transcription.

Figure 32. Nucleotide sequence of the *dhfr*IV dihydrofolate reductase showing translation of amino acids numbered from the initial methionine residue.

• • • • indicates the ribosomal binding site.

-35b **-35a** **-10b**

TAACTGAACA CGCGCTTAAT GAAGCCGGCATTAATTCATC

-10a • • • • Met Ile Arg

CAACCCTAAACT CCATTAAGGAAACATC ATG ATT CGT

						10			
Met	*Ile*	*Leu*	*Ala*	*Ile*	*Asn*	*Asn*	*Gln*	*Cys*	*Phe*
ATG	ATT	CTT	GCA	ATT	AAC	AAC	CAA	TGC	TTT
						20			
Ile	*Gly*	*Lys*	*Asn*	*Asn*	*Thr*	*Leu*	*Met*	*Tyr*	*Arg*
ATC	GGT	AAA	AAC	AAC	ACA	CTG	ATG	TAT	CGT
						30			
Leu	*Lys*	*Asp*	*Asp*	*Met*	*Leu*	*Asn*	*Phe*	*Lys*	*Lys*
TTG	AAA	GAC	GAT	ATG	TTG	AAC	TTC	AAA	AAG
						40			
Met	*Thr*	*Gln*	*Asn*	*Asn*	*Ile*	*Val*	*Val*	*Met*	*Gly*
ATG	ACA	CAA	AAC	AAC	ATC	GTG	GTG	ATG	GGG
						50			
Arg	*Lys*	*Thr*	*Phe*	*Glu*	*Ser*	*Leu*	*Asn*	*Asn*	*Arg*
CGT	AAA	ACA	TTT	GAG	TCG	TTG	AAT	AAT	CGC
						60			
Gly	*Leu*	*Pro*	*Asn*	*Arg*	*Leu*	*Asn*	*Val*	*Val*	*Val*
GGG	TTA	CCT	AAT	CGA	CTA	AAC	GTG	GTG	GTG

70

Thr	*Ser*	*Lys*	*Ala*	*Glu*	*Thr*	*Phe*	*Glu*	*Asp*	*Ile*
ACA	TCA	AAA	GCG	GAA	ACG	TTT	GAA	GAC	ATT

80

Gln	*Thr*	*Ile*	*Thr*	*Thr*	*His*	*Asp*	*Met*	*Lys*	*Arg*
CAG	ACC	ATC	ACA	ACC	CAT	GAC	ATG	AAA	CGT

90

Ser	*Glu*	*Thr*	*Phe*	*Thr*	*Lys*	*Glu*	*Gly*	*His*	*Val*
TCT	GAA	ACC	TTT	ACC	AAA	GAA	GGT	CAT	GTC

100

Val	*Tyr*	*Ile*	*Thr*	*Pro*	*Asp*	*Ser*	*Phe*	*Ile*	*Asn*
GTC	TAC	ATC	ACT	CCT	GAT	AGC	TTT	ATC	AAT

110

Gln	*Phe*	*Leu*	*Pro*	*Phe*	*His*	*Arg*	*Asp*	*Ser*	*Glu*
CAG	TTT	CTT	CCT	TTT	CAT	CGT	GAC	AGC	GAA

120

Asp	*Glu*	*Ile*	*Trp*	*Val*	*Ile*	*Gly*	*Gly*	*Trp*	*Arg*
GAT	GAA	ATC	TGG	GTC	ATT	GGT	GGT	TGG	CGC

130

Arg	*Tyr*	*Met*	*Arg*	*Gln*	*Pro*	*Leu*	*His*	*Val*	*His*
AGG	TAT	ATG	AGG	CAG	CCA	CTC	CAT	GTG	CAT

140

Lys	*Ser*	*Phe*	*Val*	*His*	*Leu*	*Trp*	*Met*	*Met*	*Thr*
AAA	TCA	TTT	GTA	CAT	TTG	TGG	ATG	ATG	ACG

Arg	*End*	
AGG	TGA	TGTAGCGCTAAACTAACTCTTTGGCGG

Homology between the nucleotide sequences of the *dhfr*IV and the *E. coli* K12 chromosomal gene is 37% over the first 210 residues. Over the first 146bp, homology was greatest with the gene for the chromosomal DHFR, *fol*A, of *Klebsiella aerogenes*, at 51% (comparisons made with the EMBL database); the homology with the *E. coli fol*A over this region was 47%.

Table 52. Homologies between the DHFR type IV amino acid sequence and other plasmid-encoded DHFRs.

DHFR	Over whole enzyme		First 70 residues only	
	% Homology	% Similarity	% Homology	% Similarity
Chromosomal	26	38	37	54
type IIIa	26	30	43	54
type IIIc	28	36	46	57
type IX	19	30	30	47
type I	30	32	34	47
type V	24	33	37	51
type VI	20	31	33	50
type VII	22	32	36	50

A search using the SWISSPROT database revealed homologies with the DHFRs of other species at the protein level. However this was only at the N-terminal sequence. The DHFR of *Bacillus subtilis* is 33%, homologous 56% similar, over the first 78 residues. *Lactobacillus casei* showed 43% identity, 63% similarity, over a region from amino acid 13 to 67. The DHFRs of *Enterococcus faecium* and *Neisseria gonorrhoeae* gave similar levels of homology over various regions at the N-terminal sequence. The percentage homologies and similarities over the entire protein and the first 70 amino acids are summarised in table 52. The DHFRs show greatest homology at the N-terminal sequence (table 52); when conservative amino acid changes are considered, the similarity between the type IV and the other plasmid-mediated trimethoprim-resistant DHFRs is approximately 50% in this region. This figure falls to just over 30% if the entire sequence is considered. The type IV DHFR is most like the type IIIc in sequence, being 57% similar over the first 70 amino acids and 36% similar over the entire gene (table 52).

CHAPTER 6

DISCUSSION

Very high rates of carriage of gram-negative lactose-fermenting rods resistant to the commonly used antimicrobials, chloramphenicol, trimethoprim and ampicillin, were recorded in the normal faecal flora of the population in the town of Vellore and three surrounding villages. Almost all of the sample population carried bacteria resistant to these antibiotics. As such, these results demonstrate the highest recorded incidence of carriage of antibacterial resistant organisms in the normal faecal flora of a population. In studies of trimethoprim resistance, for example, resistance in *E. coli* causing infections has been shown in the west usually to be below 20% (Heikkilä *et al* 1990a; Amyes *et al* 1986; Towner and Slack 1986). Even in special locations such as geriatric and long-stay hospitals, resistance to trimethoprim has not approached the level found in the present study. Bendall *et al* (1989) showed 50% resistance in a geriatric ward in the UK; Heikkilä *et al* (1990b) showed 40% in a similar situation in Finland, whilst Amyes *et al* (1986) showed 64% resistance in urinary tract isolates in a long-stay unit. In developing countries, higher levels of resistance have been recorded; for example 63.3% of urinary tract isolates were found to be resistant to trimethoprim in Nigeria (Lamikanra and Ndep 1989), and in Vellore resistance in urinary tract isolates was shown to be 64% (Young *et al* 1986a). In studies of normal flora in the Sudan, 96% resistance to ampicillin and 77% resistance to trimethoprim was recorded in faecal coliforms from children (Shears *et al* 1988). In two studies in the Netherlands, high levels of resistance in faecal flora were recorded; 89% resistance to ampicillin and 28% resistance to trimethoprim were the highest figures recorded (Bonten *et al* 1990; 1992).

In direct comparisons of developed countries to developing countries higher rates of carriage of resistant organisms have been recorded in normal faecal

gram-negative rods; for example, in a sample of children in Boston, only 1 in 39 carried a trimethoprim resistant organism whilst 25 of 41 in Peru and 34 of 53 in China carried trimethoprim resistant *E. coli.* (Lester *et al* 1990). Although only small sample numbers were used, this does give an indication of the scale of the problem in the developing world.

This study demonstrated almost universal carriage of resistant coliforms in the normal faecal flora, and this must influence the resistance rates seen in enteric pathogens. Of the trimethoprim resistant wild-type strains, 35% were capable of transferring trimethoprim resistance to the standard *E. coli* K12; of these most were capable of transferring trimethoprim resistance, along with resistance to any other antimicrobials situated on the plasmids, to a strain of *Salmonella typhimurium.* Thus, the resistance plasmids are capable of transfer to an enteric pathogen and expressing the resistance genes. It, therefore, seems feasible that transfer of these resistance mechanisms is possible to other enteric pathogens in the wild, as has been demonstrated by Griffin *et al* (1989) who identified a plasmid mediating resistance to trimethoprim-sulphamethoxazole in a *Shigella* outbreak as having originated in an *E. coli* from a urinary tract infection.

Such high rates of resistance in the normal flora are reflected in high rates of resistance in pathogenic isolates. In this region high levels of resistance are seen in pathogenic isolates. In isolates from urinary tract infections, resistance to trimethoprim was found to be high, at 64% (Young *et al* 1986a). Reports have also been made of high levels of resistance to trimethoprim amongst *Shigella flexneri* isolates, 84% resistant, and *S. shigae*, 88% resistant, in Vellore (Jesudason *et al* 1989). Transferable resistance to trimethoprim has also been seen in *Vibrio cholerae* in this area (Jesudason and John 1990).

What are the possible contributory factors in this high rate of carriage of faecal organisms resistant to these antimicrobials? A high level of consumption of antibiotics is an obvious source for the promotion of resistance in all categories of bacteria. In India there is very little control over the use of antibiotics; in general the only constraint is perhaps financial. Many of the commonly used antimicrobials, such as trimethoprim, co-trimoxazole, chloramphenicol, nalidixic acid and ampicillin, can be bought from the many pharmacies without prescription, although by law these are necessary to obtain antimicrobials. This results in

self-dosing, probably with incorrect doses since the tablets may be bought singly and many of the people are very poor; possibly for inappropriate reasons when bacterial infection is not the cause or the wrong agents are used for the causative infecting organism. The bacteria, particularly commensal bacteria, are therefore exposed to sub-optimal doses of antimicrobial which promotes the development of resistance mechanisms (Thomson and Amyes 1993) and the maintenance and spread of these mechanisms in the bacterial population via plasmids (Anderson *et al* 1973; Petrocheilou *et al* 1977) and possibly transposons and integrons.

This is not, however, the only factor contributing to the propagation and maintenance of antibiotic resistant bacteria in the faecal flora. General hygiene and living conditions in Third world areas are low and this must promote the transmission of faecal bacteria. High levels of faecal bacteria were found to contaminate the drinking water of both the urban and rural populations; these bacteria were also very often resistant to antimicrobial agents and carried transferable resistance plasmids (Young and Jesudason 1990). Cramped living conditions without proper facilities and a population perhaps ignorant of basic hygiene must also contribute to the transmission of resistant organisms.

The high incidence of illness and enteric disease, in particular, that is evident in the population of this study, is also of importance. The causative bacteria of the enteric diseases that are prevalent in this region are, by their very nature infectious, and unhygienic and poor living conditions obviously support their transmission and any resistance genes that they may carry. If resistance can be transferred from commensals to pathogens then the opposite is likely to be true. Thus, a cyclical promotion of resistance genes is possible between commensals and pathogens, encouraged by an ever-present challenge with antimicrobials at sub-optimal concentrations.

The resistance to nalidixic acid seen in this study perhaps illustrates some of these points. Resistance to nalidixic acid is at a much lower rate than resistance to the other antibacterials, trimethoprim, ampicillin and chloramphenicol. Nalidixic acid resistance, unlike resistance to the other three agents, is never plasmid-mediated. Thus, transmission of resistance between strains via plasmid-transfer and the subsequent movement of other mobile genetic elements that have been shown to contribute to the distribution of resistance genes is not possible. A difference

between the urban and rural populations in their carriage of nalidixic acid resistant organisms was also recorded but was not observed in resistance to any of the other antibacterials. The urban population had significantly higher rates of carriage of nalidixic acid resistance than was shown in the rural population. This is perhaps the result of the greater use of nalidixic acid by the urban population in the treatment of shigellosis; thus the organisms in the town are exposed to the drug more frequently, with the subsequent increase in resistance rates. The non-transferable nature of the resistance means that resistance to nalidixic acid is more limited and the rates of carriage do not reach the levels seen with the other antimicrobials.

The transferable, plasmid-mediated nature of trimethoprim resistance in this study no doubt contributed to the levels of resistance to this agent seen in this population. Approximately 35% of trimethoprim resistance was mediated by plasmids capable of transfer by conjugation. Most of these plasmids were capable of transfer, replication and expression of resistance in *Salmonella typhimurium*. They are then capable of mediating resistance in a known enteric pathogen, although proving a direct link between resistance in commensal organisms and pathogens may be difficult, this provides evidence that there is the potential for such a link.

The plasmids mediating trimethoprim resistance were not of one or even a few types, but many different plasmids were responsible for resistance, showing a diverse population of resistance plasmids capable of spreading and maintaining both themselves and the resistance mechanisms that they harbour in the commensal and pathogenic enterobacteria. Many of these plasmids also carried resistance mechanisms to other agents, and a few could be characterized as multiresistance plasmids. This provides for possible co-selection of diverse resistance mechanisms situated on the same plasmid so that treatment with one agent not only selects for resistance to that agent but also to those other agents located on the plasmid (Amyes 1989). Sulphamethoxazole resistance is present on most of the plasmids, rendering them resistant to the commonly used trimethoprim-sulphamethoxazole combination. The TEM-1 β-lactamase, mediating resistance to ampicillin, was also identified in all of those plasmids mediating resistance to ampicillin, of which there were 25. Worldwide, this is the most frequently identified of the plasmid-mediated β-lactamases and is the progenitor of many of the extended spectrum β-lactamases that are responsible for resistance to the third generation cephalosporins. The change from TEM-1 to the extended spectrum β-lactamases involves only a very

few mutations (Payne and Amyes 1991). The potential is therefore present in the plasmid population for rapid evolution to resistance to the third generation cephalosporins if the challenge of the use of these agents becomes frequent.

Resistance to the first generation cephalosporin, cephaloridine, and to the amoxycillin/clavulanic acid combination was also seen to be mediated by TEM-1 in a few of the plasmids. This was probably by hyper-production of the enzyme which produces low level resistance to these agents. As was later shown, the induction of TEM-1 associated with those plasmids harbouring the type IV DHFR was also responsible for resistance to amoxycillin/clavulanic acid that was not identified initially.

Fortunately no resistance was seen to the third generation cephalosporins or gentamicin; this is perhaps because these are very expensive drugs and are delivered by the I.V. route and are therefore limited mostly to hospital use. If the use of the newer oral cephalosporins were to become more common, the potential for the rapid evolution of resistance mechanisms is present in the form of TEM-1. Resistance to these agents was also absent from the original wild-type isolates. The original isolates did, however, have a wide-range of other resistance to other agents, most of the strains being resistant to sulphamethoxazole, ampicillin, tetracycline and streptomycin. Many of the wild-types were multiresistant, being resistant to six or more antibacterial agents.

Half of the plasmid-mediated trimethoprim resistance resulted from the presence of the *dhfr*V gene. This gene, mediating high level resistance to trimethoprim, has been found in many locations around the world, although its prevalence is not as high, in other locations, as that of the *dhfr*I. It was, however, first isolated in Sri Lanka, which is very close to Tamil Nadu where this study was conducted. In Sri Lanka also it was shown to be the most common cause of plasmid-mediated trimethoprim resistance. This study was also conducted on the commensal faecal flora, although only in children (Sundström *et al* 1987). There are thus two possibilities, either the *dhfr*V is more prevalent in this part of the world or is more common in normal faecal flora. Unfortunately there is no information on its prevalence in pathogenic isolates in this area, although in other studies of normal faecal flora in the US the *dhfr*V is not commonly found. It may be, then, that the *dhfr*V is more common in plasmids in this part of the world. When the original

wild-type strains were tested for the presence of the *dhfr*I and the *dhfr*V, the *dhfr*I was shown to be very much more common in strains that were not capable of transferring resistance to trimethoprim. In total, 37% of trimethoprim resistance was shown to be mediated by the *dhfr*I gene, while approximately 22% was mediated by the *dhfr*V. However, only 10% of this resistance was as the result of transferable *dhfr*I but 18% of resistance was due to transferable *dhfr*V. The 27% of trimethoprim resistance as a result of "non-transferable" *dhfr*I is a likely consequence of the transposition of Tn*7*, carrying the *dhfr*I gene, onto the *E. coli* chromosome. The transposon is preferentially inserted into a locus on the *E. coli* chromosome (Lichtenstein and Brenner 1982) and this has been shown to be an increasing trend in trimethoprim resistance (Towner 1982; Kraft *et al* 1986; Heikkilä *et al* 1991).

In total, 35% of trimethoprim resistance was as the result of transferable plasmid-mediated enzymes and a further 30% was as the result of identifiable non-transferable resistance genes, most of which were *dhfr*I.

The transposon and integron systems associated with the Tn*21*-like transposons were also shown to be present, although some of the plasmids were shown not to possess a fully functional transposon. Positive hybridisation with the probe for the *tnp*A transposase gene was not obtained in eight of the plasmids carrying the *dhfr*V, indicating the absence of the transposase. All the *dhfr*V genes were, however, associated with the integrase-like ORF of Tn*21* responsible for integration of "gene-cassettes" into the Tn*21*-like group of transposons (Sundström and Sköld 1990). Whether those genes not associated with the *tnp*A gene had undergone direct integration into the plasmid by the action of the integron/integrase, as is the case in the type II genes encoded by plasmids R751 and R388 (Sundström *et al* 1988) or that the *tnp*A had been deleted was not established. It is interesting to note that those plasmids possessing a *tnp*A gene had, in general, more resistance mechanisms associated with them, whereas those without the transposase gene had fewer, in four cases only the trimethoprim resistance determinant, perhaps indicating integration into the plasmid without the presence of the transposon, which commonly has more than one resistance mechanism associated with it. Further examination of the plasmids would be necessary to confirm this.

In those plasmids harbouring the *dhfr*I gene, all carried the ORF for the integrase of the Tn*7* transposon, the transposon usually associated with the *dhfr*I gene. Spectinomycin resistance is also usually carried by this transposon. In those plasmids isolated in this study, however, three are sensitive to spectinomycin. In these cases rearrangements or deletions, possibly mediated by the integrase, may have resulted in the loss of the resistance phenotype, or the replacement of spectinomycin resistance with another resistance gene, as has been recorded previously (Tietze *et al* 1987; Sundström *et al* 1991).

In seven plasmids, all responsible for low level resistance to trimethoprim, the *dhfr*IV gene was identified as mediating trimethoprim resistance. The *dhfr*IV was first characterized in *E. coli* isolated from urinary tract infections from Vellore in 1984 (Young *et al* 1986b). Since then it has not been identified in any other study and this is the first report of the DHFR type IV since the initial report. Five of the plasmids harbouring the gene were shown to be identical in both their resistance profile and their restriction endonuclease digest pattern; these plasmids were all isolated from samples from the same village. It might be expected that since both studies were conducted in the same area that the same resistance mechanism might be found; however, the *dhfr*IV mediates only very low level resistance, as assayed by commonly used methods (MIC 4mg/L) and would appear to be a fairly inefficient resistance mechanism when compared to enzymes such as the types I and V. It has survived this competition; thus the enzyme's unique property amongst the DHFRs of being inducible may be important in the resistance produced by this enzyme.

The induction mechanism was shown to be present, although one plasmid was capable of mediating higher levels of resistance by constitutive expression of moderate levels of the enzyme. The plasmid pUK2007 was shown to be inducible, and as had been shown in the plasmids reported previously, the enzyme was expressed in increased amounts when the cells harbouring it were challenged with increasing concentrations of trimethoprim. Thomson *et al* (1993) have shown that this is a metabolic effect that is abolished by the presence of thymidine in the growth medium. Thymidine, being the ribonucleoside of thymine, is an antagonist of trimethoprim, which acts by depleting thymine in the cell, producing thymine starvation and subsequent thymine-less death (Amyes and Smith 1974a).

It was shown in this study that deprivation of thymidine in a thymine requiring auxotroph of *E. coli* J62-2 carrying the plasmid pUK2007 resulted in the increased expression of the DHFR type IV. Thus, thymine-less stress is sufficient for the induction of the DHFR type IV and the metabolic basis for the production of the induction response by trimethoprim.

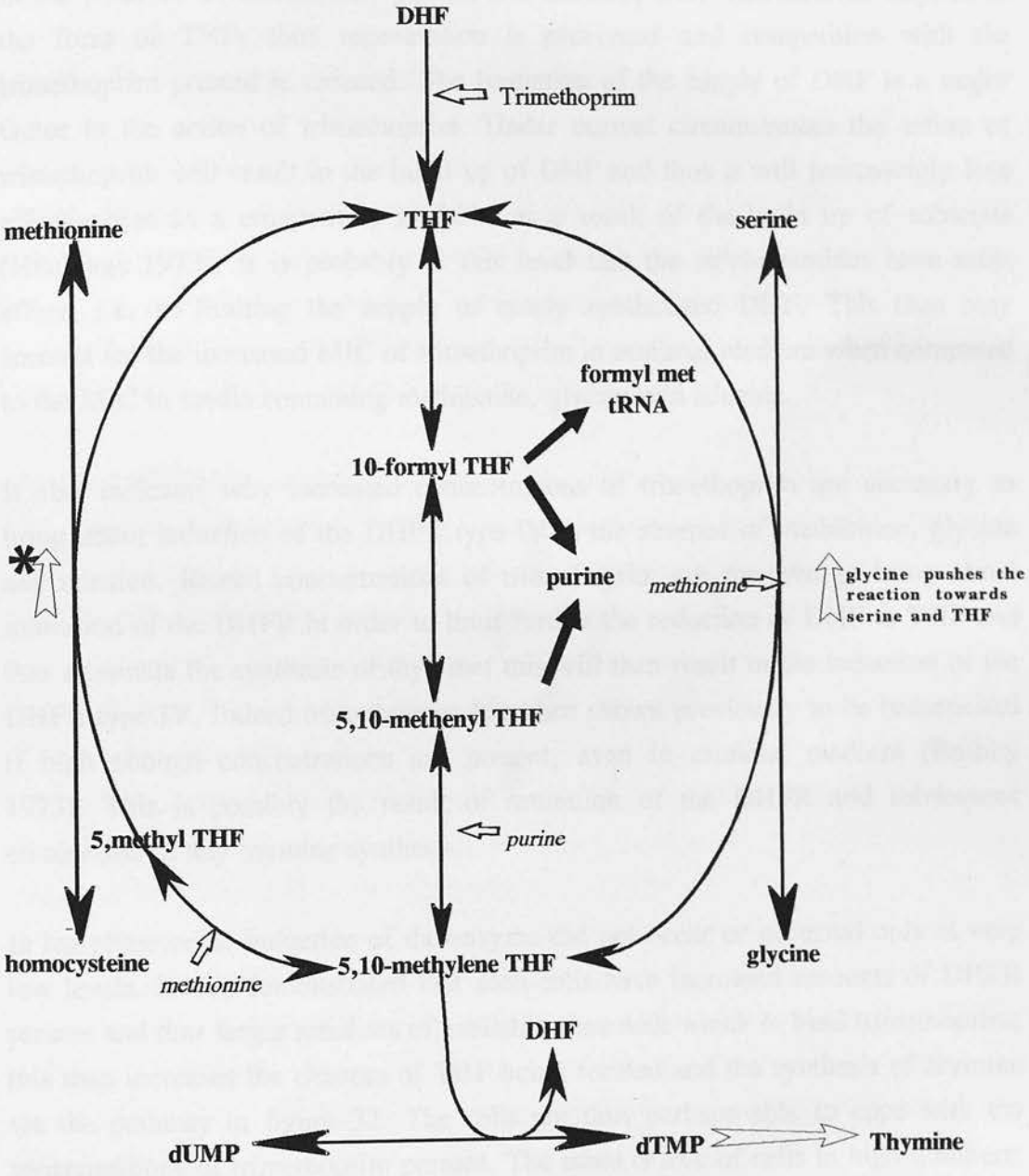
Thomson *et al* (1993) speculated that the presence of methionine, glycine and adenine was necessary for the induction of the DHFR type IV to occur. This was presumed because at 40mg/L no induction of the enzyme is produced when these metabolites are not present in the media. Amyes and Smith (1974a) had also previously shown that these metabolites are necessary, because of their role in the DHF - thymine pathway (figure 32) conserving the THF pool by acting to inhibit its conversion to 10-formyl THF, a necessary step towards the formation of thymine (figure 32), to bring about thymineless cell death. It has been shown here, however, that increased concentrations of trimethoprim will bring about induction of the DHFR type IV in the absence of methionine, glycine and adenine. Increased concentrations of trimethoprim were tried initially because it was shown that in minimal medium, the MIC is considerably higher than in media containing methionine, glycine and adenine. So given that significantly higher concentrations of trimethoprim than the MIC, as derived by the agar dilution method on IST agar, were necessary to produce induction it seemed that because higher MICs are obtained in minimal media higher concentrations of trimethoprim would be necessary to produce induction. The higher MICs and the higher concentration required to bring about induction of the type IV, as will be seen, both reflect the involvement of the pathway shown below in which DHF is reduced to THF then regenerated in the production of thymine.

Increased concentrations of trimethoprim are required to bring about induction of the DHFR type IV in the absence of the metabolites, methionine, glycine and adenine. Thymine production is prevented in the presence of methionine, glycine and adenine, by the inhibitory effect of these metabolites on the reactions after the formation of THF. Methionine, glycine and adenine act to conserve the THF pool which is depleted by the action of trimethoprim on the dihydrofolate reductase. This then results in depletion of the precursors, specifically 5,10 methylene THF, that lead to thymine formation, and therefore thymine depletion and subsequent cell death. This also has the effect of halting the regeneration of DHF, further

contributing to the action of trimethoprim which is a competitive analogue of DHF. Thus rapid thymine starvation occurs and induction of the DHFR takes place.

Although THF production is inhibited some will be produced; because trimethoprim is a competitive inhibitor of dihydrofolate reductase there is a statistical likelihood that some reduction of DHF will take place. This will be dependant upon the K_i of the enzyme, i.e. the sensitivity of the enzyme to trimethoprim, the concentration of trimethoprim, the concentration of DHF available and the amount of enzyme present. If methionine, glycine and adenine are not present the THF which is produced may be enough to maintain thymine production, which would in the presence of methionine, glycine and adenine have been prevented by their inhibition of the formation of 5,10-methylene THF. In minimal medium, growth and protein synthesis will be slower; protein synthesis being a necessary factor in thymine-less death this will result in cell survival and possible continued slow growth. In the presence of the type IV DHFR which is moderately resistant to trimethoprim, and will be produced in larger amounts even without induction, being encoded as it is by a plasmid, enough THF may be produced to allow thymine synthesis and regeneration of DHF.

Figure 32. The pathway of 5,10-methylene THF formation from THF on which methionine, glycine and adenine act to produce thymine starvation in the presence of trimethoprim



The regeneration of DHF will contribute to the resistance of the cell to the effects of trimethoprim, competing as it will with the available trimethoprim, contributing to the maintenance of thymine synthesis and thus raising the MIC of trimethoprim. In the presence of methionine, glycine and adenine, DHF will become trapped in the form of THF; thus regeneration is prevented and competition with the trimethoprim present is reduced. The limitation of the supply of DHF is a major factor in the action of trimethoprim. Under normal circumstances the action of trimethoprim will result in the build up of DHF and thus it will increasingly lose effectiveness as a competitive inhibitor as a result of the build up of substrate (Hitchings 1973). It is probably at this level that the sulphonamides have most effect, i.e. in limiting the supply of newly synthesized DHF. This then may account for the increased MIC of trimethoprim in minimal medium when compared to the MIC in media containing methionine, glycine and adenine.

It also indicates why increased concentrations of trimethoprim are necessary to bring about induction of the DHFR type IV in the absence of methionine, glycine and adenine. Raised concentrations of trimethoprim are required to bring about saturation of the DHFR in order to limit further the reduction of DHF to THF and thus eliminate the synthesis of thymine; this will then result in the induction of the DHFR type IV. Indeed trimethoprim has been shown previously to be bactericidal if high enough concentrations are present, even in minimal medium (Bushby 1973). This is possibly the result of saturation of the DHFR and subsequent elimination of any thymine synthesis.

In log-phase cells, induction of the enzyme did not occur or occurred only at very low levels. It was demonstrated that such cells have increased amounts of DHFR present and thus larger numbers of available sites with which to bind trimethoprim; this then increases the chances of THF being formed and the synthesis of thymine via the pathway in figure 32. The cells are thus perhaps able to cope with the concentrations of trimethoprim present. The same is true of cells in high numbers; greater cell numbers will mean larger numbers of DHFR molecules will be available to "soak-up" the trimethoprim present and THF formation will again be more likely. The effect of inoculum size on MICs has been documented. Bushby (1973) observed up to 300-fold increase in MIC when the inoculum was raised from 5 organisms to $5x10^5$ organisms, and no doubt this is the same process at work. Thus, in log-phase cells induction is abolished, despite high concentrations

of trimethoprim. However, it may be that other factors are also involved; log-phase cells may contain more thymine or DHF or both. It would seem reasonable that in rapidly dividing cells higher concentrations would be present, not only of DHFR, but also of thymine and DHF. The presence of increased concentrations of these factors and the enzyme would act to decrease the effect of trimethoprim and reduce the induction of the DHFR type IV.

This may go some way to explaining the route whereby trimethoprim produces a metabolic effect which results in the increased expression of the DHFR type IV. It was, however, shown that the gene for the TEM-1 β-lactamase was present on the plasmid that harboured the *dhfr*IV, and that this mechanism of resistance was also inducible. This has never before been demonstrated in TEM-1 or any other plasmid-mediated β-lactamase in gram-negative bacteria.

The inducible chromosomal β-lactamases possessed by many species of gram-negative rod are induced as a direct response to the antibiotic, although intervening cell wall breakdown products may act as intermediates (Korfmann and Sanders 1989), with a response being seen almost immediately on addition of the antibiotic to the medium in log-phase cells. This is not true of the mechanism described here; the response to the antibiotic is not seen in log-phase cells or cells in high numbers, as was seen to be true of the response to trimethoprim. High concentrations of ampicillin, far in excess of the MIC, were necessary to produce the induction response, as was also shown in the response of the DHFR type IV to trimethoprim. The high concentrations of antibiotic are probably necessary to swamp the available TEM-1 and thus allow ampicillin to take effect. However, much lower levels are necessary when clavulanic acid is present to inhibit the β -lactamase. Thus, it would also seem likely that the induction of both the TEM-1 and the DHFR type IV by the β-lactam antibiotics described is a result of a metabolic stress brought about by the action of the drugs, as is the case with trimethoprim.

The most surprising aspect of this mechanism is that the β-lactam drugs are able to produce induction of the DHFR type IV and that trimethoprim is able to produce induction of the β-lactamase.

The increased effectiveness of trimethoprim in the presence of methionine, glycine and adenine is also apparent in the increased expression of the β-lactamase, as would be expected in a response triggered by the metabolic stress imposed by trimethoprim. The presence of methionine, glycine and adenine has no effect on the ability of amoxycillin/clavulanic acid to produce induction of either the TEM-1 or the DHFR type IV.

The role of thymidine was, however, more difficult to assess. Thymine starvation produced induction of both mechanisms and thus a general metabolic response producing induction of both mechanisms is implicated. The β-lactams and trimethoprim have little in common and it is difficult to imagine a mechanism that could link them other than a general metabolic response to the stress brought about by the treatment with these antimicrobials. It was noted that neither treatment with nalidixic acid nor streptomycin was capable of producing a similar response. However, nalidixic acid resistance is not present on the plasmid and hence no induction of a resistance mechanism is possible that would permit recovery of the cells. Streptomycin, on the other hand, is an inhibitor of protein synthesis which may be necessary for the induction response.

In treatment with both amoxycillin/clavulanic acid and trimethoprim the morphology of the cells changed, and in a similar way. The cells elongate and become much larger, before they recover and grow normally. Cells treated with amoxycillin/clavulanic acid also displayed a bulge in the centre of the elongated cells. This is often seen in cells treated with β-lactam agents, and may be an intermediate in the formation of spheroplasts. This change in cell morphology was associated with concomitant cell death after inoculation of the antimicrobial containing media. The resumption of normal cell growth must be brought about when sufficient levels of enzyme have been induced to circumvent the effect of the drug in the media.

Thus a general response would seem to be implicated, perhaps involving the whole plasmid. A close link between the genes of the two resistance mechanisms could not be demonstrated by the failure to co-select recombinants resistant to both agents after restriction digest and cloning. This combined with the finding that the resistance mechanisms are not increased proportionally would seem to indicate that they are not transcribed from the same promoter. It might be possible to select

constitutive over-expressing mutants of one enzyme and show that the other is not over-expressed; this may assist in ruling-out transcription of both genes from a common promoter. Dorman (1991) has demonstrated the modification of gene expression as a result of metabolic stress on bacterial cells effecting supercoiling of plasmid DNA which in turn modifies gene expression. Such a mechanism is possibly acting here. However, the failure to clone the mechanism makes investigation of the molecular basis of this mechanism difficult, although the possibility of a *trans* acting suppressor seems to have been eliminated.

Although the molecular mechanism of induction is unclear, the phenotypic effect has been characterized. When the cells were challenged before the MIC assays were performed, a dramatic increase in the MIC of trimethoprim, ampicillin and amoxycillin/clavulanic acid was observed, and the MICs observed in broth assays, with enzyme induction, were also very much higher. So under normal circumstances the enzymes are not induced on agar plates. The resistance to trimethoprim was increased to levels comparable with high level resistance mechanisms such as the DHFRs I and V. Resistance to amoxycillin/clavulanic acid is increased to levels which may be clinically significant and result in a failure of therapy.

Such a resistance mechanism has important implications. It has been reported previously that transferable low level resistance mechanisms to trimethoprim may be overlooked (Towner and Pinn 1981). In this mechanism resistance to both trimethoprim and amoxycillin/clavulanic acid is low as assessed by normal assay conditions. When hyper-production of the enzymes occurs, resistance is significantly increased. It may be important to monitor low level resistance to indicated the levels of such mechanisms and assess the significance of low level resistance producing failure of therapy.

The structural gene of the *dhfr*IV that mediates resistance to trimethoprim is 432 base pairs and gives a translation product of M_r 17 195. This is of a size similar to other *dhfr* genes, both plasmid mediated and the chromosomal genes of many species. It does not, however, agree with the findings of Thomson, Young and Amyes (1990) that the DHFR type IV had a M_r of 33 000 as given by SDS-PAGE, under even the most strenuous of de-naturing conditions. The amino acid sequence given by the translation of the nucleotide sequence when compared to that given by

the partial N-terminal sequence (Thomson, Young and Amyes 1990) shows only one difference which occurs at position 12. Automatic N-terminal amino acid sequencing determined a threonine residue at this position but a cysteine residue was shown at this position by nucleotide sequencing. This may be significant in the structure of the DHFR type IV enzyme. Cysteine residues are rarely substituted for other amino acids without specific cause, because of their role in the conformation of proteins (Dayhoff *et al* 1978), and no other DHFR possesses a cysteine at this position. Given that the M_r is 33 000 as determined by SDS-PAGE and 17 200 by the nucleotide sequence it would seem likely that the protein occurs as a dimer and that the sub-units are joined by a di-sulphide bridge formed by the cysteine residues at position 12. So even under de-naturing conditions the sub-units would not be separated and the molecular weight of only the dimer could be determined.

Table 53. Homologies between the DHFR type IV amino acid sequence and other plasmid-encoded DHFRs.

DHFR	Over whole enzyme		First 70 residues only	
	Homology	Similarity	Homology	Similarity
E. coli chromosomal	26	38	37	54
type IIIa	26	30	43	54
type IIIc	28	36	46	57
type IX	19	30	30	47
type I	30	32	34	47
type V	24	33	37	51
type VI	20	31	33	50
type VII	22	32	36	50

At the level of the nucleotide sequence the gene is 47% homologous to the *E. coli* chromosomal gene (Smith and Calvo 1980) over the first 210 nucleotides. However, homology in the amino acid sequence over the same region was only 37%, similarity being 54%, so many of the changes in the nucleotide sequence must be significant and result in a difference in the amino acid sequence. The greatest homology at the level of the amino acid sequence was with the chromosomal DHFR of *Klebsiella aerogenes*, being 51% homologous to the

DHFR type IV, but again only at the N-terminal sequence. The type IV DHFR amino acid sequence shows homology with other DHFRs, both plasmid encoded genes and the chromosomal DHFRs of diverse species; however, this is seen only at the N-terminal sequence, for approximately the first 70 amino acids. Beyond this point comparisons could not be made. The DHFR IV has a region of some 45 amino acids, from amino acid 70 to 115, that bears no resemblance to the other DHFRs in which this region is usually shorter, approximately 20 residues long. In this region, between the NADPH binding site at amino acids 63-66 and the NADPH/trimethoprim binding site at positions 115-119, the other DHFRs all show very much reduced homology; this area seems not to be associated with any specific function and this may account for the reduced homology.

The DHFR type IV is rather unusual in that this region is somewhat extended. This may have resulted from the insertion of a section of DNA from another location. Only at the NADPH/trimethoprim binding site associated with the amino acids in the region 119-124 is there any homology at the C-terminal end. The type IV DHFR is somewhat shorter after this region than is seen in the other DHFRs, stretching only 19 residues whereas other DHFRs are approximately 40 residues longer than this and form another NADPH binding site which seems to be completely absent in the *dhfr*IV gene.

When comparing the translation to amino acid sequence of the nucleotide sequence with the other DHFR amino acid sequences before and after amino acid 70, the lack of homology after amino acid 70 is revealed. For instance, the similarity between the DHFR IV and the *E. coli* chromosomal gene is 54% over the first 70 amino acids but only 38% over the entire gene, and in the type IIIc the similarity is 57% over the first 70 amino acids but only 36% over the entire protein, while the similarity in the IIIa drops in these regions from 54% to 30%. Thus it would seem that the type IV has been conserved in the N-terminal region but is very unlike the other DHFRs at the C-terminal end, except around the NADPH/trimethoprim binding site. Since the N-terminal region is most heavily associated with enzyme function (Rouch *et al* 1989) it seems reasonable that the C-terminal region can undergo considerable change without affecting the function of the enzyme. This raises the question of how to compare sequences and assess degrees of relatedness between proteins; certainly in this case it would seem inappropriate to take the percentages for homology over the entire gene as reflecting the DHFR type IV's

relationship to other DHFRs since this would demonstrate a very low degree of relatedness that would not reflect the considerable homology demonstrated at the N-terminal end, which is more important for enzyme function. The considerable differences in the C-terminal region may be associated with the induction mechanism; however, this may be unlikely since TEM-1 is also inducible in this plasmid, and from the pI derived for this enzyme it would not seem to be considerably different from other TEM-1 enzymes, so inducibility is most likely associated with structures outwith the resistance genes themselves.

If the N-terminal region only is considered, the type IV is most similar to the type IIIc, the IIIa, the *E. coli* chromosomal DHFR and the *K. aerogenes* chromosomal DHFR. It is significant that the type IV is most similar to chromosomal and low level trimethoprim resistance DHFRs since this enzyme is only moderately resistant to inhibition by trimethoprim.

If the amino acid sequences of the type IV, other plasmid-mediated DHFRs, and the *E. coli* chromosomal DHFR (figure 33) are aligned, considerable conservation of homology can be seen at sites associated with the function of the enzyme, i.e. with NADPH binding sites, methotrexate and trimethoprim binding sites that are analogous with DHF binding sites. There are, however, a number of changes identifiable in these regions that may be associated with changes in function or resistance of the enzyme. It is, however, difficult to identify specific changes that result in an increase in resistance to trimethoprim, as can be done in the TEM enzymes such that a change in single amino acids has been identified with a change in specificity and function of the enzyme (Payne and Amyes 1991). The differences in the DHFRs are more gross and the effect of a difference in single amino acids can only be speculated upon. There are changes in sequence that may be identified at important regions and may be significant in changes of enzyme function. Often these differences are conservative between similar amino acids, and such changes are often consistent between different DHFRs. The type IV also has a number of changes in amino acids that are normally highly conserved in the other DHFRs (figure 33).

Figure 33. Amino acid sequence of the DHFR type IV compared with other DHFR amino acid sequences from the *E. coli* chromosomal (Chr) enzyme and plasmid mediated enzymes.

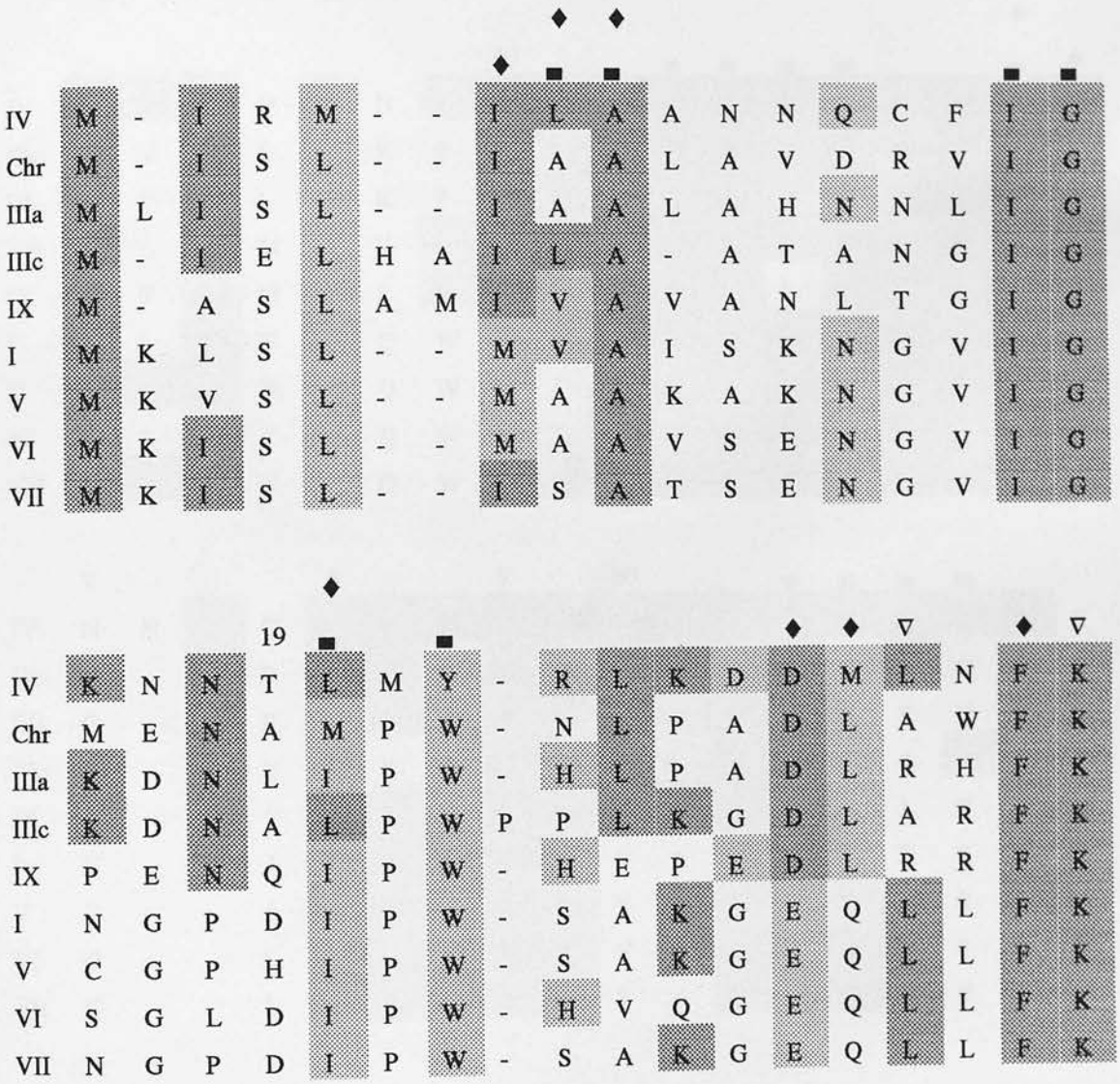


Figure 33. Amino acid sequence of the DHFR type IV compared with other DHFR amino acid sequences from the *E. coli* chromosomal (Chr) enzyme and plasmid mediated enzymes, continued.

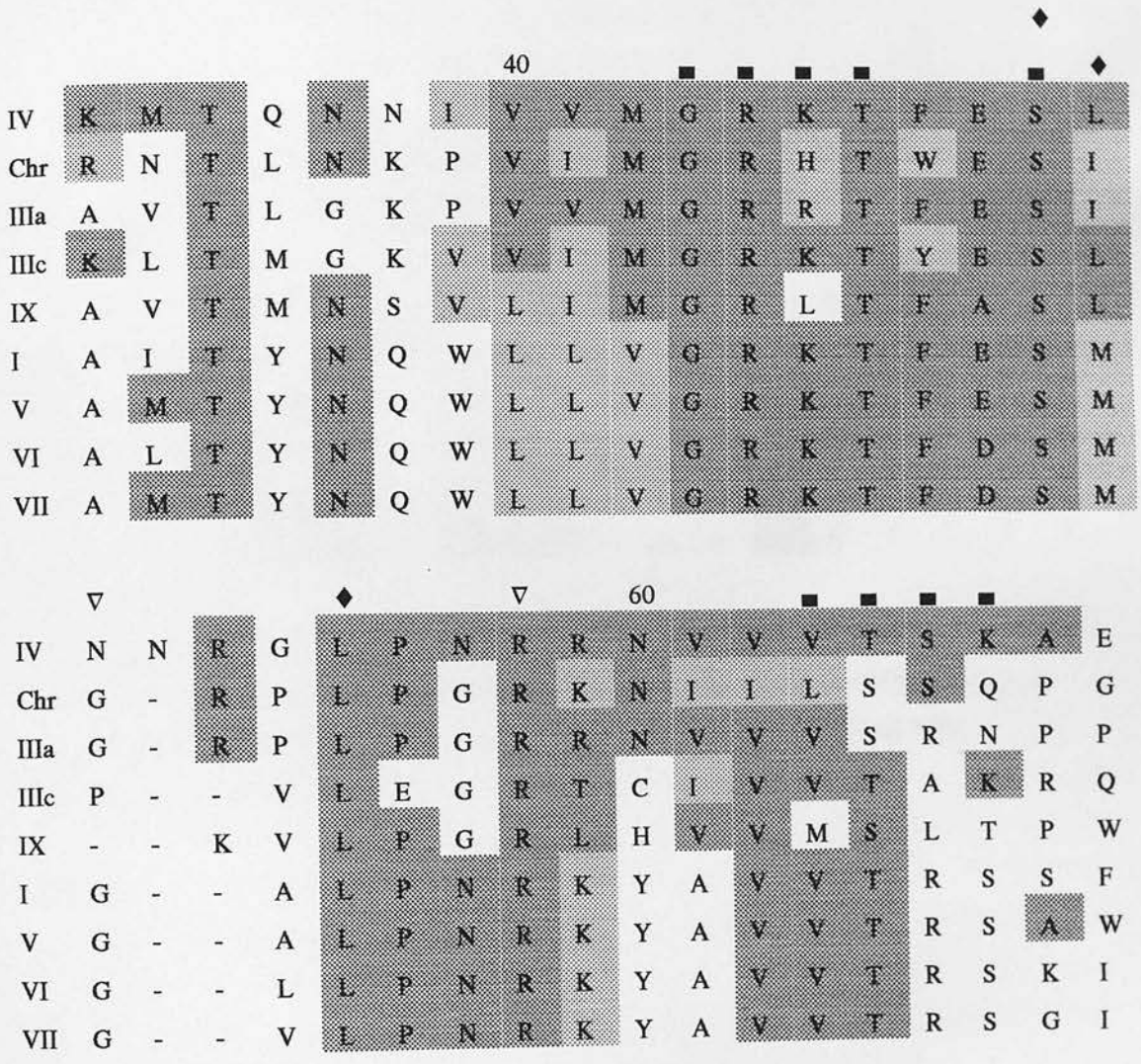
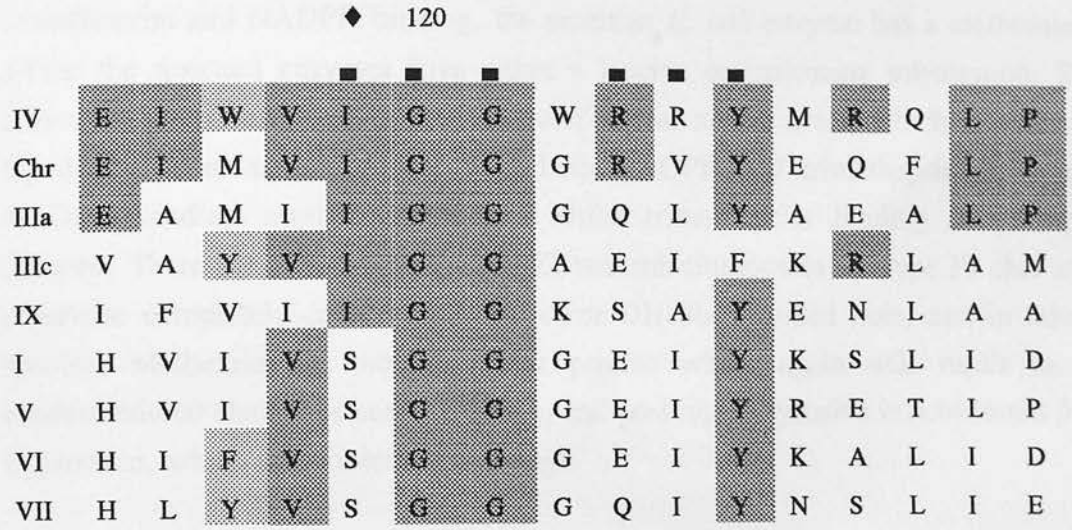


Figure 33. Amino acid sequence of the DHFR type IV compared with other DHFR amino acid sequences from the *E. coli* chromosomal (Chr) enzyme and plasmid mediated enzymes, continued.

No important comparisons can be made until the region around amino acid 120.



■ Indicates an amino acid associated with an NADPH binding site.

♦ Indicates an amino acid associated with a trimethoprim binding site.

∇ Indicates an amino acid associated with a methotrexate binding site.

(Rouch *et al* 1989)

G

Shaded residues are homologous.

Y

Shaded residues are similar.

At amino acids 5-7, associated with an NADPH and trimethoprim binding site, the sequence is conserved, although at position 6 there is some variation. At position 7 alanine is completely conserved. The complete conservation of isoleucine and glycine at positions 14 and 15 is seen in all the plasmid-encoded DHFRs and across species, this again being involved in NADPH binding. From position 18 to 22,

associated with both NADPH and trimethoprim-DHF binding, several interesting differences are seen. At position 18, although not directly associated with a binding site but perhaps close enough to exert an influence, the low level resistance enzymes and the *E. coli* chromosomal enzyme have an asparagine residue, the high level resistance mechanisms having a proline or leucine. Proline, in particular, may be important since it will cause a change in strand direction, because of the residues shape, and thus cause a change in conformation perhaps altering trimethoprim binding. Then at position 20, which is directly associated with both trimethoprim and NADPH binding, the sensitive *E. coli* enzyme has a methionine whilst the resistant enzymes have either a leucine or isoleucine substitution. So although there is a difference it is between similar amino acids, which is perhaps significant at an area associated with both NADPH and trimethoprim binding; NADPH binding must be preserved whilst trimethoprim binding is perhaps reduced. There are at positions 21 and 22 two substitutions in the type IV that are otherwise completely conserved in the other DHFRs included here, and in other species. Methionine is substituted for proline which again will result in a conformational change as outlined above, and position 22 tyrosine is substituted for tryptophan, which is a conservative change.

The region from amino acids 27 to 32 is a region which has been associated with methotrexate, trimethoprim and thus DHF binding (Rouch *et al* 1989). The aspartate residue has been implicated as an important residue in differentiating between trimethoprim resistant and sensitive DHFRs (Sundström *et al* 1993). In the sensitive and low level resistance mechanisms the aspartate is conserved, as it is in the chromosomal enzymes of other species. In the highly resistant DHFRs, however, the aspartate is substituted for a glutamic acid residue. This residue is also seen in mammalian DHFRs which are resistant to the action of trimethoprim. Although this is a conservative substitution, these amino acids being very similar, glutamic acid has a larger side-chain and this may be the significant difference, preventing trimethoprim binding by steric hinderance. Also at position 28 in the low level resistance mechanisms either leucine or methionine, similar amino acids, are present but in highly resistant DHFRs a glutamine is found. The residues phenylalanine and lysine are highly conserved at positions 31 and 32, indicating a significant role in DHF binding.

From position 40 stretching to 51 there is a region that is concerned with NADPH binding and, towards the end of this region, with trimethoprim binding. Within this region is a highly conserved glycine, arginine, lysine, threonine, phenylalanine motif (although conservative substitutions of lysine with histidine or arginine and phenylalanine for tryptophan or tyrosine are found). This highly conserved region is also present in the DHFR type IV. Serine at position 50 is also highly conserved, being directly involved with both NADPH and trimethoprim binding; however, at position 51 there are conservative substitutions with possible significance in trimethoprim binding, i.e. leucine or methionine for isoleucine.

The leucine, proline residues of positions 55 and 56, and the arginine at position 58 are highly conserved. At position 63, although there is involvement with NADPH binding, there is consistent substitution of leucine in the chromosomal *E. coli* enzyme, as well as the chromosomal DHFRs of other species, for a valine residue in the resistant plasmid-mediated DHFRs; this is a conservative substitution which may have implications for enzyme function, being consistent throughout many genes encoding trimethoprim resistant dihydrofolate reductases. There then follows the region of reduced homology in which it is not feasible to make comparisons between the type IV and the other DHFRs.

At the region associated with NADPH and trimethoprim binding at amino acids 119-125, homology is again seen when the sequence of the type IV is aligned with the other DHFRs. The glycine doublet which is entirely conserved throughout the DHFRs is present in the DHFR type IV, and over the length of this short region the type IV resembles both the *E. coli* chromosomal enzyme and the other plasmid-mediated DHFRs. After this region, homology once again disappears and the DHFR ends earlier than the other DHFRs.

Many of the changes mentioned above may affect function and therefore resistance conferred by the enzyme. This, however, can only be speculation as effects on the binding as it relates to specific amino acid changes of the two substrates, NADPH and DHF, and trimethoprim, are very difficult to understand. Mutation studies or site directed mutagenesis would have to be undertaken in order to investigate the effect of single changes on enzyme function and sensitivity to trimethoprim.

In the region immediately upstream of the structural gene of the *dhfr*IV two possible -10 and -35 promoter regions were identified, along with a single ribosomal binding site terminating 9bp upstream of the initiation codon, ATG.

Of the two possible -10 and -35 promoter regions, one of the -10 regions (TAAACT) was identified 12bp upstream of the ribosomal binding site and the complementary -35 (CCGGCA) region was taken as being 17bp upstream of the -10 region, as a 17bp separation is the most common between these promoter regions (Harley and Reynolds 1987). Neither of these sites showed a high degree of homology with the *E. coli* consensus promoter sequences as shown by Harley and Reynolds (1987), and as such are possibly weak promoters.

The other -10 and -35 promoter regions that were identified were further upstream than those described above. The -10 site (TTCATC) starts 28bp from the ribosomal binding sequence and a possible -35 region (CACGCG) was identified 20bp upstream of the -10 sequence. Although this is not ideal spacing between the two promoter regions, such separation is seen and these possible promoters have more in common with the *E. coli* consensus promoter sequences (Harley and Reynolds 1987) than those described above. This may, however, represent only a weak promoter of the *dhfr*IV gene. The weak similarity to *E. coli* promoters may reflect a possible role in the induction mechanism; an investigation of the promoters of the other DHFR type IV gene that did not show induction, that of plasmid pUK2026, may indicate the part played by these promoter regions in induction. The dissimilarity between the promoter region of the *dhfr*IV gene and the *E. coli* consensus promoters may also reflect the origins of the enzyme in a species unrelated to *E. coli* or indeed the *Enterobacteriaceae*.

CONCLUSIONS

There was almost universal carriage of gram-negative rods resistant to the commonly used antimicrobial agents, ampicillin, chloramphenicol and trimethoprim in the normal faecal flora of the population in and around the town of Vellore, in Tamil Nadu.

It could be speculated that this was as a result of poor hygiene and general living conditions combined with the indiscriminate use of antimicrobials. This presents a threat to the continued use of these antimicrobials and presents a basis for the evolution of novel resistance mechasisms.

Transferable trimethoprim resistance was mediated by a number of plasmids that were also responsible for resistance to a range of other antimicrobial agents. The predominant enzyme responsible for plasmid-encoded trimethoprim resistance was the type V DHFR, and this is an unusual finding. When non-transferable resistance is also considered, however, the *dhfr*I gene is the most prevalent trimethoprim resistance gene, probably as a result of transposition into the *E.coli* chromosome, as has been widely reported by other workers. This makes the resistance gene more stable, being less likely to be lost from the chromosome but remaining available for transposition into any plasmid entering the cell.

In all the plasmids that encoded the DHFR type I, the transposon Tn*7* was also identified; this is the most frequently found location for the *dhfr*I gene. In all of those plasmids that harboured the *dhfr*V, gene the integrase-like ORF of Tn*21* was present, but in several plasmids the *tnp*A gene of the Tn21 transposase was not identified, indicating that Tn*21* was not present in a fully functional form.

The *dhfr*IV was identified in all of those plasmids that mediated low level trimethoprim resistance. The inducible nature of this resistance mechanism was shown to be a result of thymine starvation imposed on the cell by the action of trimethoprim. The plasmid on which the *dhfr*IV was characterized also carried the

TEM-1 β-lactamase. As part of this plasmid the TEM-1 β-lactamase was also shown to be inducible; such a response has never been demonstrated previously in this enzyme. Trimethoprim and the β-lactam agents, amoxycillin and amoxycillin/clavulanic acid, were capable of producing induction of both the DHFR type IV and the TEM-1 β-lactamase.

It was speculated that this was perhaps a general response to the metabolic stress imposed on the cell by these agents; however, a mechanism to account for this could not be established. This response did, however, produce dramatic increases in resistance to trimethoprim and amoxycillin/clavulanic acid that would not normally be detected by commonly used assay methods for determining antimicrobial sensitivities.

The *dhfr*IV is related to the other plasmid mediated *dhfr* genes and to the chromosomal gene, *fol*A, of *E. coli* and other species, both gram-positive and gram-negative. Homology was greatest at the active sites that have been identified in these enzymes. There were, however, great differences in sequence towards the C-terminal end that perhaps indicates a genetic origin, of this unusual DHFR, removed from the *Enterobacteriaceae*.

BIBLIOGRAPHY

ABRAHAM, E.P. (1983). History of β-lactam antibiotics. HANDBOOK OF EXPERIMENTAL PHARMACOLOGY, **67/1**, 1-14.

ABRAHAM, E.P., CHAIN, E. (1940). An enzyme from bacteria able to destroy penicillin. *Nature (London)*, **146**, 837.

ACAR, J.F., GOLDSTEIN, F.W. (1982). Genetic aspects and epidemiologic implications of resistance to trimethoprim. *Rev. Infect. Dis.*, **4**, 270-275.

ACAR, J.F., GOLDSTEIN, F.W., GERBAUD, G.R., CHABBERT, M.A. (1977). Plasmides de resistance au trimethoprime et groupes d'incompatibilite. *Ann. Inst. Pasteur (Microbiologie)*, **128A**, 41-47.

AGODI, A., JONES, C., THRELFALL, E.J., D'ANGELO, M., MARRANZANO, M. (1990). Molecular characterization of trimethoprim resistance in *Shigella sonnei* in Sicily. *Epidemiol. Infect.*, **105**, 29-40.

AKIBA, T. et al (1960). On the mechanism of the development of multiple-drug resistant clones of *Shigella*. *Jpn J. Microbiol.*, **4**, 219-217. As cited in WATANABE, T., FUKUSAWA, T. (1960). "Resistance transfer factor" an episome in *Enterobacteriaceae. Biochem. Biophys. Res. Commun.*, **3**, 660-665.

AMYES, S.G.B. (1989). The success of plasmid encoded resistance in clinical bacteria. *J. Med. Microbiol.*, **28**, 73-83.

AMYES, S.G.B., DOHERTY, C.J., WANNACOTT, S. (1986). Trimethoprim and Co-trimoxazole: a comparison in their use in respiratory tract infections. *Scand. J. Infect. Dis.*, **18**, 561-566.

AMYES, S.G.B., DOHERTY, C.J., YOUNG, H-K. (1986). High level trimethoprim resistance in urinary tract bacteria. *Eur. J. Clin. Microbiol.*, **5**, 287-291.

AMYES, S.G.B., SMITH, J.T. (1974a). Trimethoprim action and its analogy with thymine starvation. *Antimicrob. Agents Chemother.*, **5**, 169-178.

AMYES, S.G.B., SMITH, J.T. (1974b). R-factor trimethoprim resistance mechanism: An insusceptible target site. *Biochem. Biophys. Res. Commun.*, **58**, 412-418.

AMYES, S.G.B., SMITH, J.T. (1975). Thymineless mutants and their resistance to trimethoprim. *J. Antimicrob. Chemother.*, **1**, 85-89.

AMYES, S.G.B., TAIT, S. (1990). Trimethoprim resistance in Staphylococci. *J. Med. Microbiol.*, **31**, 4-7.

AMYES, S.G.B., TOWNER, K.J. (1990). Trimethoprim resistance; epidemiology and molecular aspects. *J. Med. Microbiol.*, **31**, 1-19.

AMYES, S.G.B., TOWNER, K.J., CARTER, G.I., THOMSON, C.J., YOUNG, H-K. (1989). The type VII dihydrofolate reductase: a novel plasmid-encoded trimethoprim resistant enzyme from gram-negative bacteria isolated in Britain. *J. Antimicrob. Chemother.*, **24**, 111-119.

AMYES, S.G.B., TOWNER, K.J., YOUNG, H-K. (1992). Classification of plasmid-encoded dihydrofolate reductases conferring trimethoprim resistance. *J. Med. Microbiol.*, **36**, 1-3.

ANDERSON, J.D. (1974). The effect of R-factor carriage on the survival of *Eschericia coli* in the human intestine. *J. Med. Microbiol.*, **7**, 85-90.

ANDERSON, J.D., GILLESPIE, W.A., RICHMOND, M.H. (1973). Chemotherapy and antibiotic-resistance transfer between enterobacteria in the human gastro-intestinal tract. *J. Med. Microbiol.*, **6**, 461-473.

ANDREWS, P. (1964). Estimation of the molecular weight of proteins by sephadex gel-filtration. *Biochem. J.*, **91**, 222-223.

BACHMANN, B. (1972). The pedigrees of some mutant strains of *Eschericia coli*. *Bacteriol. Rev.*, **36**, 525-555.

BANNATYNE, R.M., TOMA, S., CHEUNG, R., HU, G. (1980). Resistance to trimethoprim and other antibiotics in Ontario *Shigellae*. *Lancet*, **1**, 425-426.

BARADA, F.A., GUERRANT, R.L. (1980). Sulphamethoxazole-trimethoprim versus ampicillin in treatment of acute invasive diarrhea in adults. *Antimicrob. Agents Chemother.*, **17**, 961-964.

BARG, N.L., HUTSON, F.S., WHEELER, L.A., et al (1990). Novel dihydrofolate reductases isolated from epidemic strains of trimethoprim-sulphamethoxazole-resistant *Shigella sonnei*. *J. Infect. Dis.*, **162**, 466-473.

BARTH, P.T., DATTA, N., HEDGES, R.W., GRINTER, N.J. (1976) Transposition of a deoxyribonucleic acid sequence encoding trimethoprim and streptomycin resistances from R483 to other replicons. *J. Bacteriol.*, **125**, 800-810.

BARTOLOMÉ, B., JUBETE, Y., MARTINEZ, E., de la CRUZ F. (1991). Construction and properties of a family of pACYC184 derived cloning vectors compatible with pBR322 and its derivatives. *Gene*, **102**, 75-78.

BENDALL, M.J., EBRAHIM, S., FINCH, R.G., SLACK, R.C.B., TOWNER, K.J. (1989). Bacterial resistance to trimethoprim in geriatric medical wards. *Gerontology*, **35**, 121-126.

BENNET, P.M., LINTON, A.H. (1986). Do plasmids influence the survival of bacteria? *J. Antimicrob. Chemother.*, **18**(suppl. C), 123-126.

BERGAN, T., BRODWALL, E.K. (1972). Human pharmacokinetics of a sulphamethoxazole-trimethoprim combination. *Acta Med. Scand.*, **192**, 483-492.

BERTINO, J.B., STACEY, K.A. (1966). A suggested mechanism for the procedure for isolating thymine requiring mutants of *Eschericia coli. Biochem. J.*, **101**, 32c-33c.

BISSONNETTE, L., ROY, P.H. (1992). Characterization of In0 of *Pseudomonas aeruginosa* plasmid pVS1 an ancestor of integrons of multiresistance plasmids and transposons of gram-negative bacteria. *J. Bacteriol.*, **174**, 1228-1257.

BONTEN, M., STOBBERINGH, E., PHILIPS, J., HOUBEN, A. (1990). High prevalence of antibiotic resistant *Eschericia coli* in faecal samples of students in the south-east of the Netherlands. *J. Antimicrob. Chemother.*, **26**, 585-592.

BONTEN, M., STOBBERINGH, E., PHILIPS, J., HOUBEN, A. (1992). Antibiotic resistance of *Eschericia coli* in faecal samples of healthy people in two different areas in an industrialized country. *Infection*, **20**, 258-262.

BROWN, G.M. (1962). The biosynthesis of folic acid. II Inhibition by sulphonamides. *J. Biol. Chem.*, **237**, 536-540.

BROWN, G.M. (1971). The biosynthesis of pteridines. *Advan. Enzymol.*, **35**, 35-77.

BROWN, N. L., WINNIE, J.N., SEIFF, M., SILVER, S. (1986). The nucleotide sequence of the mercuric resistance operons of plasmid R100 and transposon Tn501 further evidence for *mer* genes which enhance the mercuric ion detoxification system. *Mol. Gen. Genet.*, **202**, 143-151.

BRUMFITT, W., HAMILTON-MILLAR, J.M.T., WOOD, A. (1983). Evidence for a slowing in trimethoprim resistance during 1981 - a comparison with earlier years. *J. Antimicrob. Chemother.*, **11**, 503-509.

BURCHALL, J.J. (1973). Mechanism of action of trimethoprim-sulfamethoxazole -II. *J. Infect. Dis.*, **128**(Suppl. 5), S437-441.

BURCHALL, J.J., ELWELL, L.P., FLING, M.E. (1982). Molecular mechanisms of resistance to trimethoprim. *Rev. Infect. Dis.*, **4**, 246-254.

BURCHALL, J.J., HITCHINGS, G.H. (1965). Inhibitor binding analysis of dihydrofolate reductases from various species. *Mol. Pharmacol.*, **1**, 126-36.

BURNS, J.L., LIEN, D.M., HEDIN, L.A. (1989). Isolation and characterization of dihydrofolate reductase from trimethoprim-susceptible and trimethoprim-resistant *Pseudomonas aeruginosa*. *Antimicrob. Agents. Chemother.*, **33**, 1247-1251.

BUSHBY, S.R.M. (1973). Trimethoprim-sulfamethoxazole: *in vitro* microbiological aspects. *J. Infect. Dis.*, **128**(Suppl. 5), S442-S462.

BUSHBY, S.R.M., HITCHINGS, G.H. (1968). Trimethoprim, a sulphonamide potentiator. *Br. J. Pharmacol.*, **33**, 72.

BUTLER, T.L., RUMUS, L., ARNOLD, K. (1982). Response of typhoid fever caused by chloramphenicol-susceptible and chloramphenicol-resistant strains of *Salmonella typhi* to treatment with trimethoprim-sulfamethoxazole. *Rev. Infect. Dis.*, **4**, 551-561.

CHANG, L-L., CHANG, S-F., CHOW, T-Y. (1992). The distribution of the DHFR genes in trimethoprim resistant urinary tract isolates from Taiwan. *Epidemiol. Infect.*, **109**, 453-462.

CHOPRA, I. (1986). Genetic and biochemical basis of tetracycline resistance. *J. Antimicrob. Chemother.*, **18**(Suppl. C), 51-56.

CHATKAEMORAKOT, A., ECHEVERRIA, P., TAYLOR, D.N., et al (1987). Trimethoprim resistant *Shigella* and enterotoxigenic *Eschericia coli* strains in children in Thailand. *Pediatr. Infect. Dis.*, **6**, 735-739.

CHIRNSIDE, E.D., EMMERSON, A.M., SMITH, J. (1985). A follow-up survey of transferable, plasmid-encoded trimethoprim resistance in a general hospital (1975-1983). *J. Antimicrob. Chemother.*, **16**, 419-434.

COLLIS, C.M., HALL, R.M. (1992) Site-specific deletion and rearrangement of integron insert genes catalysed by the integron DNA integrase. *J. Bacteriol.*, **174**, 1574-1585.

COUGHTER, J.P., JOHNSTON, J.L., ARCHER, G.L. (1987). Characterization of a staphylococcal trimethoprim resistance gene and its product. *Antimicrob. Agents Chemother.*, **32**, 1027-1032.

DARREL, J.H., GARROD, C.P., WATERWORTH, P.M. (1968). Trimethoprim, laboratory and clinical studies. *J. Clin. Pathol.*, **21**, 202-209.

DATTA, N. (1969). Drug resistance and R-factors in the bowel of London patients before and after admission to hospital. *Br. Med. J.*, **2**, 407-411.

DATTA, N., HEDGES, R.W. (1972). Trimethoprim resistance conferred by W plasmids in *Enterobacteriaecae. J. Gen. Microbiol.*, **72**, 349-355.

DATTA, N., HUGHES, V.M. (1983). Plasmids in the same inc. groups in enterobacteria before and after the medical use of antibiotics. *Nature* (London), **306**, 616-617.

DATTA, N.G., KONTIMICHALOU, P. (1965). Penicillinase controlled by infectious R-factors in *Enterobacteriaceae. Nature* (London), **208**, 239-241.

DAVIS, B.D., MINGIOLI, E.S. (1950). Mutants of *Eschericia coli* requiring methionine or vitamin B12. *J. Bacteriol.*, **60**, 17-28.

DAYHOFF, M.O., SCHWARTZ, R.M., ORCUTT, B.C. (1978). A model of evolutionary change in proteins. In ATLAS OF PROTEIN SEQUENCE AND STRUCTURE (M.O. Dayhoff ed.) National Biomedical Research Foundation, Washigton D.C. Volume 5, supplement 3, pp345-352.

DEGROOT, R. CHAFFIN, D.O., KUEHN, M., SMITH, A.L. (1991). Trimethoprim resistance in *Haemophilus influenzae* is due to altered dihydrofolate reductase. *Biochem. J.*, **15**, 657-662.

DICKERMAN, H.W. (1971) The role of folate coenzymes in the inhibition of protein synthesis. *Ann. N. Y. Nat. Acad. Sci.*, **186**, 70-81.

DOMAGT, G. (1935). Ein beitrag zur chemotherapie der bakteriellen infektion. *Deutsche Medezinische Wachenschrift*, **61**, 250-253.

DORMAN, C.J. (1991). DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. *Infect. Immun.*, **59**, 745-749.

ELWELL, L.P., FLING, M.E. (1989). Resistance to trimethoprim. pp249-290. In HANDBOOK IN EXPERIMENTAL PHARMACOLOGY, L.E. Bryan (ed). **91**, *Resistance to Drugs.*

FARRAR, W.E. (1985). Antibiotic resistance in developing countries. *J. Infect. Dis.*, **152**, 1103-1106.

FLEMING, A. (1929). On the antibacterial action of cultures of a *Penicillium* with special reference to their use in the selection of B. influenzae. *Br. J. Exp. Path.*, **10**, 226-236.

FLEMING, M.P., DATTA, N., GRUNEBERG, R.N. (1972). Trimethoprim resistance determined by R-factors. *Br. Med. J.*, **1**, 726-728.

FLENSBURG, J., SKÖLD, O. (1987). Massive over-production of dihydrofolate reductase in bacteria as a reponse to the use of trimethoprim. *Eur. J. Biochem.*, **162**, 473-476.

FLENSBURG, J., STEEN, R. (1986). Nucleotide sequence analysis of the trimethoprim resistant dihydrofolate reductase encoded by R-plasmid R751. *Nuc. Acids Res.*, **14**, 5933.

FLING, M.E., ELWELL, L.P. (1980). Protein expression in *Eschericia coli* minicells containing recombinant plasmids specifying trimethoprim resistant dihydrofolate reductase. *J. Bacteriol.*, **141**, 779-785.

FLING, M.E., KOPF, J., RICHARDS, C. (1988). Characterization of plasmid pAZ1 and the type III dihydrofolate reductase gene. *Plasmid*, **19**, 30-38.

FLING, M.E., RICHARDS, C. (1983). The nucleotide sequence of the trimethoprim-resistant dihydrofolate reductase gene harbored by Tn*7*. *Nuc. Acids Res.*, **11**, 5147-5158.

FLING, M.E., WALTON, L., ELWELL, L.P. (1982). Monitoring of plasmid-encoded, trimethoprim-resistant dihydrofolate reductase genes: Detection of a new resistant enzyme. *Antimicrob. Agents Chemother.*, **22**, 882-888.

FORNASINI, M., REVES, R.R., MURRAY, B.E., et al (1992). Trimethoprim resistant *Eschericia coli* in households of children attending day-care centres. *J. Infect. Dis.*, **166**, 326-330.

FREISHEIM, J.H., BITAR, K.G., REDDY, A.V., BLANKENSHIP, D.T. (1978). Dihydrofolate reductase from amethopterin-resistant *Lactobacillus casei*: Sequences of the cyanogen bromide peptides and the complete sequences of the enzyme. *Antimicrob. Agents Chemother.*, **33**, 1247-1251.

FROST, J.A., ROWE, B., VANDEPITTE, J. (1982). Aquisition of trimethoprim resistance in epidemic strain of *Shigella dysenteriae* 1 from an epidemic in central Africa. *J. Hyg.*, **94**, 163-172.

GALAS, D.J., CHANDLER, M. (1981). On the molecular mechanisms of transposition. *Proc. Nat. Acad. Sci.* (USA), **78**, 4858-4862.

GOLDBERGER, R.F. (1974). Autogenous regulation of gene expression. *Science*, **183**, 810-816.

GOLDSTEIN, F.W., ACAR, J.F. (1990). Evolution and spread of trimethoprim resistance in gram-negative bacteria. *J. Med. Microbiol.*, **31**, 1-4.

GOLDSTEIN, A., ARONOW, L., KALMAN, S. (1974). Drug resistance. In PRINCIPLES OF DRUG ACTION: THE BASIS OF PHARMACOLOGY. John Wiley and Sons.

GOLDSTEIN, F.W., GERBAUD, G., COURVALIN, P. (1986). Transposable resistance to trimethoprim and 0129 in *Vibrio cholerae*. *J. Antimicrob. Chemother.*, **17**, 559-569.

GOLDSTEIN, F.W., PAPADOPOLOU, B., ACAR, J.F. (1986). The changing pattern of trimethoprim resistance in Paris, with a review of worldwide experience. *Rev. Infect. Dis.*, **8**, 725-737.

GREENWOOD, D. (1979). Relevance of *in vitro* synergy to therapy, does synergy between diaminopyrimidines and sulphonamides operate at concentrations achievable in the urine. *J. Antimicrob. Chemother.*, **5**(suppl. C), 85-89.

GREENWOOD, D., O'GRADY, F. (1976). Activity and action of trimethoprim and sulphamethoxazole against *Escherichia coli*. *J. Clin. Path.*, **29**, 162-166.

GREY, D. HAMILTON-MILLER, J.M.T., BRUMFITT, W. (1979). Incidence and mechanism of resistance to trimethoprim in clinically isolated gram-negative bacteria. *Chemotherapy*, **25**, 147-156.

GRIFFIN, P.M., TAUXE, R.V., REDD, S.C. et al (1989). Emergence of highly trimethoprim-sulfamethoxazole resistant *Shigella* in a native American population: An epidemiological study. *Am. J. Epidemiol.*, 129, 1042-1051.

GRINDLEY, N.D.F., REED, R.R. (1985). Transpositional recombination in prokaryotes. *Ann. Rev. Biochem.*, **54**, 838-896.

GRINSTEAD, J. (1986). Evolution of transposable elements. *J. Antimicrob. Chemother.*, **18**(Suppl. C), 77-84.

GRINSTEAD, J., de la CRUZ, F., SCHMITT, R. (1990). The Tn*21* subgroup of bacterial transposable elements. *Plasmid*, **24**, 163-169.

GROSS, R.J., THRELFALL, E.J., WARD, L.R., ROWE, B. (1984). Drug resistance in *Shigella dysenteriae, S. flexneri and S. boydii* in England and Wales: Increasing incidence of resistance to trimethoprim. *Br. Med. J.*, **288**, 784-786.

GRUNEBERG, R.W. (1984). Antibiotic senstivities of urinary pathogens 1971-1982. *J. Antimicrob. Chemother.*, **14**, 17-25.

GUTMANN, L., WILLIAMSON, R., MOREAU, N., *et al* (1985). Cross-resistance to nalidixic acid, trimethoprim and chloramphenicol associated with alterations in outer-membrane proteins of *Klebsiella, Enterobacter* and *Serratia*. *J. Infect. Dis.*, **151**, 501-507.

HAIDER, K., CHATKAEMORACOT, A., KAY, B.A. (1990). Trimethoprim resistance in *Shigella dysenteriae* I isolates obtained from widely scattered locations of Asia. *Epidemiol. Infect.*, **104**, 219-228.

HALL, R.M., VOCKLER, C. (1987). The region of the inc N plasmid R46 coding for resistance to β-lactam antibiotics, streptomycin/spectinomycin and sulphonamide is closely related to resistance segments found in inc W plasmids and in Tn*21*-like transposons. *Nuc. Acid. Res.*, **15**, 7491-7501.

HAMILTON-MILLAR, J.M.T. (1979). Mechanisms and distribution of bacterial resistance to diaminopyrimidines and sulphonamides. *J. Antimicrob. Chemother.*, **5**(Suppl. B), 61-73.

HAMILTON-MILLAR, J.M.T., PURVES, D. (1986). Trimethoprim resistance and trimethoprim usage in and around the Royal Free Hospital in 1985. *J. Antimicrob. Chemother.*, **18,** 643-644.

HANAHAN, D. (1983). Studies on transformation of *Eschericia coli* with plasmids. *J. Mol. Biol.*, **166**, 557-580.

HARLEY, C.B., REYNOLDS, R.P. (1987). Analysis of *Eschericia coli* promoter sequences. *Nuc. Acids Res.*, **15**, 2343-2361.

HARNETT, N. (1992). High level resistance to trimethoprim, co-trimoxazole and other antimicrobial agents among clinical isolates of *shigella* spp. in Onatrio, Canada -an update. *Epidemiol. Infect.*, **109**, 463-472.

HARSHEY, R.M., BUKHARI, A.J. (1981) A mechanism of DNA transposition. *Proc. Nat. Acad. Sci.* (USA), **78**, 1090-1094.

HARTMANN, P.G., STÄHLI, M., KOCHER, H.P., THEN, R.L. (1988). N-terminal amino acid sequence of a chromosomal dihydrofolate reductase purified from trimethoprim-resistant *Staphylococcus aureus*. *FEBS Lett.*, **242**, 157-160.

HAWKEY, P.M. (1986). Resistant bacteria in the normal human flora. *J. Antimicrob. Chemother.*, **18**(Suppl. C), 133-139.

HAYEK, L.J., NETHERWAY, J. (1976). Thymine requiring bacteria. *Lancet*, **1**, 1080.

HEDGES, R.W. (1987). A plasmid of group Q which confers resistance to trimethoprim and sulphonamides. *Microbiol. Immunol.*, **31**, 1113-1115.

HEDGES, R.W., DATTA, N.G., FLEMING, P. (1972). R-factors conferring resistance to trimethoprim but not sulphonamides. *J. Gen. Microbiol.*, **73**, 573-575.

HEDGES, R.W., DATTA, N.G., KONTIMICHALOU, P., SMITH, J.T. (1974). Molecular specificities of R-factor determined B-lactamases: Correlation with plasmid compatability. *J. Bacteriol.*, **117**, 56-62.

HEDGES, R.W., JACOB, A.E. (1974). Transposition of ampicillin resistance from RP4 to other replicons. *Mol. Gen. Genet.*, **132**, 31-40.

HEIKKILÄ, E., RENKONEN, O. V., SUNILA, R., UURASMAA, P., HUOVINEN, P. (1990a). The emergence and mechanisms of trimethoprim resistance in *Eschericia coli* isolated from outpatients in Finland. *J. Antimicrob. Chemother.*, **25**, 275-283.

HEIKKILÄ, E., SIITONEN, M., JAHKLA, *et al* (1990b). Increase of trimethoprim resistance among *Shigella* species, 1975-1988: analysis of resistance mechanisms. *J. Infect. Dis* ., **161**, 1242-1248.

HEIKKILÄ, E., SUNDSTRÖM, L., HUOVINEN, P. (1990c). Trimethoprim resistance in *Eschericia coli* isolates from a geriatric unit. *Antimicrob. Agents Chemother.*, **34**, 2013-2015.

HEIKKILÄ, E., SUNDSTRÖM, L., SKURNIK, M., HUOVINEN, P. (1991). Analysis of genetic localization of the type I trimethoprim resistance gene from *Eschericia coli* isolated in Finland. *Antimicrob. Agents Chemother.*, **35**, 1562-1569.

HITCHINGS, G.H. (1969). Species differences among dihydrofolate reductases as a basis for chemotherapy. *Post-Grad. Med.*, **45**(Suppl. C), 7.

HITCHINGS, G.H. (1973). Mechanisms of action of trimethoprim-sulphamethoxazole. *J. Infect. Dis.*, **128** (Suppl. 5), S1-S4.

HITCHINGS, G.H. (1983). Functions of tetrahydrofolate and the role of dihydrofolate reductase in cellular metabolism. In INHIBITION OF FOLATE METABOLISM CHEMOTHERAPY. *Handbook of Experimental Pharmacology.*, **64**, 11-20.

HUOVINEN, P. (1987). Trimethoprim Resistance. *Antimicrob. Agents Chemother.*, **31**, 1451-1456.

HUOVINEN, P., PULKINNEN, L., HELIN, H-L., MÄKILÄ, M., TOIVANEN, P. (1986). Emergence of trimethoprim resistance in relation to drug consumption in a Finnish hospital from 1971-84. *Antimicrob. Agents Chemother.*, **29**, 73-76.

JANSSON, C., FRANKLIN, A., SKÖLD, O. (1992). Spread of a newly found trimethoprim resistance gene, *dhfr*IX, among porcine isolates and human pathogens. *Antimicrob. Agents Chemother.*, **36**, 2704-2708.

JANSSON, C., SKÖLD, O. (1991). Appearance of a new trimethoprim resistance gene, *dhfrIX*, in *Eschericia coli* from swine. *Antimicrob. Agents Chemother.*, **35**, 1891-1899.

JESUDASON, M.V., JOHN, T.J. (1990). Transferable trimethoprim resistance in *Vibrio cholerae* 01 encountered in southern India. *Trans. R. Soc. Trop. Med. Hyg.*, **84**, 136-137.

JESUDASON, M.V., JOSEPH, D., KOSHI, G. (1989). Transferable trimethoprim resistance in *Shigellae* encountered in Vellore (South India). *Indian J. Med. Res.*, **89**, 297-299.

JOBANPUTRA, R.S., DATTA, N. (1974). Transferable R-factors in Enterobacteria from clinical specimens. *J. Med. Microbiol.*, **7**, 169-178.

JOYNER, S.S., FLING, M.E., STONE, D., BACCANARI, D.P. (1984). Characterization of an R-plasmid dihydrofolate reductase with a monomeric subunit structure. *J. Biol. Chem.*, **259**, 5851-5856.

KAPLAN, S.A., WEINFELD, R.E., ABRUZZO, C.W., *et al* (1973). Pharmokinetic profile of trimethoprim-sulphamethoxazole in man. *J. Infect. Dis.*, **128**, (Suppl. 5),S547-S555.

KATZAN, H.M., BUCHANAN, J.M. (1965). Enzymatic synthesis of the methyl group of methionine. VIII Repression-derepression, purification and properties of

5,10-methylene tetrahydrofolate reductase from *Eschericia coli*. *J. Biol. Chem.*, **240**, 825-835.

KIRBY, W.M.M. (1944). Extraction of a highly potent penicillin inactivator from penicillin resistant *Staphylococci*. *Science*, **99**, 452-453.

KLECKNER, N. (1981). Transposable elements in prokaryotes. *Ann. Rev. Genet.*, **15**, 341-404.

KLOCK, G., UNGER, B., GATZ, C., *et al* (1985). Heterologous repressor-operator recognition among four classes of tetracycline resistance determinants. *J. Bacteriol.*, **161**, 326-332.

KOCH, A.L. (1981). Evolution of antibiotic resistance gene function. *Microbiol. Rev.*, **45**, 355-378.

KORFMANN, G., SANDERS, C.C. (1989). *ampG* is essential for high-level expression of AmpC β-lactamase in *Enterobacter cloacae*. *Antimicrobial. Agents Chemother.*, **33**, 1946-1951.

KORFMANN, G., WEIDEMANN, B. (1988). Genetic control of β-lactamase production in *Enterobacter cloacae*. *Rev. Infect Dis.*, **10**, 793-799.

KRAFT, C.A., TIMBURY, M.C., PLATT, D.J. (1986). Distribution and genetic location of Tn*7* in trimethoprim-resistant *Eschericia coli*. *J. Med. Microbiol.*, **22**, 125-131.

LACEY, R.W. (1979). Mechanism of action of trimethoprim and sulphonamides: Relevance to synergy. *J. Antimicrob. Chemother.*, **5**(Suppl. B), 75-83.

LACEY, R.W. (1982). Do sulphonamide-trimethoprim combinations select less resistance than the use of trimethoprim alone. *J. Med. Microbiol.*, **22**, 125-131.

LACEY, R.W., LORD, V.L., GUMASEKERA, H.K.W., *et al* (1980). Comparison of trimethoprim alone with trimethoprim-sulphamethoxazole in the treatment of respiratory and urinary tract infections with particular reference to selection of trimethoprim resistance. *Lancet*, **i**, 1270-1273.

LAFOND , M., COUTURE, F., VÉZINA, G., LAVESQUE, R.C. (1989). Evolutionary perspectives in multiresistance β-lactamase transposons. *J. Bacteriol.*, **171**, 6423-6429.

LAMIKANRA, A., NDEP, R.B. (1989). Trimethoprim in urinary tract pathogens in two Nigerian hospitals. *J. Antimicrob. Chemother.*, **23**, 151-154.

LESTER, S.C., PLA, M.D.P., WANG, F., *et al* (1990). The carriage of *Eschericia coli* resistant to antimicrobial agents by healthy children in Boston, Caracas, Venzuela and Qin Pu, China. *N. Eng. J. Med.*, **323**, 285-289.

LEVY, S.B., BURKE, J.P., WALLACE, C.K., *et al* (1987). Antibiotic use and resistance world wide. *Rev. Infect. Dis.* **9**(Suppl. C), S231-316.

LEVY, S.B., MARSHALL, B., SCHLUEDERBERG, S., *et al* (1988). High frequency of antimicrobial resistance in faecal flora. *Antimicrob. Agents Chemother.*, **32**, 1801-1806.

LICHTENSTEIN, C., BRENNER, S. (1981). Site-specific properties of Tn*7* transposition into the *Eschericia coli* chromosome. *Mol. Gen. Genet.*, **183**, 380-387.

LICHTENSTEIN, C., BRENNER, S. (1982). Unique insertion site of Tn*7* in the *Eschericia coli* chromosome. *Nature*(London), **297**, 601-603.

LINDQUIST, S., LINDBERG, F., NORMARK, S. (1989). Binding of the *Citrobacter freundii* AmpR regulator to a single DNA site provides both autoregulation and activation of the inducible *ampC* β-lactamase gene. *J. Bacteriol.*, **171**, 3746-3753.

LINEWEAVER, H., BURK, D. (1934). The determination of enzyme dissociation constants. *J. Am. Chem. Soc.*, **56**, 658-666.

LINTON, K.B., LEE, P.A., RICHMOND, M.H., GILLESPIE, W.A. (1970). Antibiotic resistance and transmissible R-factors in the intestinal coliform flora of healthy adults and children in an urban and rural community. *J. Hyg.* (Cambridge), **70**, 99-104.

LIVERMORE, D.M. (1987). Chromosomal beta-lactamases induction and stable derepression in relation to antibiotic resistance in gram-negative bacteria. in PROCEEDINGS OF BETA-LACTAMASES: CURRENT PERSPECTIVES. D.M. Livermore (ed). Theracom Ltd. 1987

MACADEN, R., BHAT, P. (1985). The changing pattern of resistance to ampicillin and co-trimoxazole in *Shigella* serotypes in Bangalore Southern India. *J. Infect. Dis.*, 152, 1348.

MAHILLON, J., LERECLUS, D. (1988). Structural and functional analysis of Tn4430: Identification of an integrase-like protein involved in the co-integrate resolution process. *EMBO. J.*, **7**, 1515-1526.

MARTINEZ, E., de la CRUZ, F. (1988). Transposon Tn*21* encodes a rec-A independent site-specific integration system. *Mol. Gen. Genet.*, **211**, 320-325.

MARTINEZ, E., de la CRUZ, F. (1990). Genetic elements involved in Tn*21* site-specific integration, a novel mechanism for the dissemination of anitibiotic resistance genes. *EMBO J.*, **9**, 1275-1281.

MASKELL, R., OKUBADEJO, O.A., PAYNE, R.H. (1976). Thymine-requiring bacteria associated with co-trimoxazole therapy. *Lancet*, **i**, 834-835.

MASKELL, R., OKUBADEJO, O.A., PAYNE, R.H., PEAD, L. (1978). Human infections with thymine-requiring bacteria. *J. Med. Microbiol.*, **11**, 33-45.

MATTHEWS, M., HARRIS, A.M., MARSHALL, M.J., ROSS, J.W. (1975). The use of analytical isoelectric focusing for the detection and identification of β-lactamases. *J. Gen. Microbiol.*, **88**, 169-178.

MATTHEWS, C.K., SCRIMGEOUR, K.G., HUENNEKENS, F.M. (1963). Dihydrofolic reductase. *Meth. Enzymol.*, **6**, 364-368.

MATTHEWS, D.A., SMITH, S.I., BACCANARI, D.P., *et al* (1986). Dihydrofolate reductase: The stereochemistry of inhibitor selectivity. *J. Biol. Chem.*, **260**, 392-399.

MAYER, K.H., FLING, M.E., HOPKINS, J.D., O'BRIAN, J.F. (1985). Trimethoprim in multiple genera of *Enterobacteriaecae* at a U.S. hospital: Spread of the type II dihydrofolate reductase gene by a single plasmid. *J. Infect. Dis.*, **151**, 783-789.

MESSING, J. (1979). A multipurpose cloning system based on the single stranded bacteriophage M13. *Recomb. DNA Bull.*, **2**, 43

MERCIER, J., LACHAPELLE, J., COUTURE, F. (1990). Structural and functional characterization of tnpI, a recombinase locus in Tn*21* and β-lactamase transposons. *J. Bacteriol.*, **172**, 3745-3757.

MINAMI, S., YOTSUJI, A., INOUE, M., MITSUHASHI, S. (1980), Induction of β-lactamase by various β-lactam antibiotics in *Enterobacter cloacae*. *Antimicrob. Agents Chemother.*, **18**, 382-385.

MIOVIC, M., PIZER, L.I. (1971). Effect of trimethoprim on macromolecular synthesis in *Eschericia coli*. *J. Bacteriol.*, **106**, 856-862.

MORISATO, D., KLECKNER, N. (1984). Transposase promotes double strand breaks and single joints at Tn10 termini *in vivo*. *Cell*, **39**, 181-190.

MURRAY, B.E., ALVARADO, T., KIM, K-H. *et al* (1985). Increasing resistance to trimethoprim-sulphamethoxazole among isolates of *Eschericia coli* in developing countries. *J. Infect. Dis.*, **152**, 1107-1113.

MURRAY, B.E., MATTHEWSON, T.T. (1990). Emergence of resistant fecal *Eschericia coli* in travellers not taking prophylactic antimicrobial agents. *Antimicrob. Agents Chemother.*, **34**, 515-518.

OKUBADEJO, O.A., MASKELL, R. (1973). Thymine-requiring mutants of *Proteus mirabilis* selected by cotrimoxazole *in vivo*. *J. Gen. Microbiol.*, **77**, 533-535.

OLIVA, B., BENNETT, P.M., CHOPRA, I. (1989). Penicillin binding protein 2 is required for induction of the *Citrobacter freundii* class 1 chromosomal β -lactamase in *Eschericia coli*. *Antimicrob. Agents Chemother.*, **33**, 1116-1117.

OSBORN, M.J., HUENNEKENS, F.M. (1958). Enzymic reduction of dihydrofolic acid. *J. Biol. Chem.*, **233**, 969-974.

OULLETTE, M., ROY, P.H. (1987). Homology of ORFs from Tn2603 and from R46 to site-specific recombinases. *Nuc. Acid. Res.*, **15**, 10055.

PALENQUE, E., OTERO, J.R., NORIEGA, A.R. (1983). High prevalence of non-epidemic *Shigella sonnei* resistant to cotrimoxazole. *J. Antimicrob. Chemother.*, **11**, 196-198.

PAPADOPOLOU, B., GERBAUD, G., COURVALIN, P., ACAR, J.F., GOLDSTEIN, F.W. (1986). Molecular epidemiology of resistance to trimethoprim in enterobacteria isolated in a Parisian hospital. *Annales de L'Institut Pasteur (Microbiologie)*, **137A**, 239-251.

PARAMASIVAN, C.N., SUBRAMANIAN, S., SHAMMAGASUNDARAM, N. (1977). Antimicrobial resistance and incidence of R-factors among *Salmonella* isolated from patients with enteric fever and other clinical conditions in Madras, India (1975-1976). *J. Infect. Dis.*, **136**, 796-800.

PARSONS, Y., HALL, R.M., STOKES, H.W. (1991). A new trimethoprim resistance gene *dhfr*X in the In7 integron of plasmid pDGO100. *Antimicrob. Agents Chemother.*, **35**, 2436-2439.

PASTEUR, L., JOUBERT, J.F. (1877). Charbun et septicémie. *Cr. Seanc. Acad. Sci.*, **85**, 101-115.

PATTISHALL, K.H., ACAR, J.F., BURCHALL, J.J., GOLDSTEIN, F.W., HARVEY, R.J. (1977). Two clinical types of trimethoprim-resistant dihydrofolate

reductase specified by R-plasmids of different incompatability groups. *J. Biol. Chem.*, **252**, 2319-2323.

PAYNE, D.J., AMYES, S.G.B. (1991). Transferable resistance to extended spectrum β-lactamases; a major threat or minor inconvenience. *J. Antimicrob. Chemother.*, **27**, 255-261.

PETROCEILOU, V., GRINSTED, J., BENNETT, P.M. (1977). Spread of a single plasmid clone to an untreated individual from a person receiving prolonged tetracycline therapy. *Antimicrob. Agents Chemother.*, **12**, 219-225.

PETROCEILOU, V., GRINSTED, J., BENNETT, P.M. (1979). Persistence of plasmid-carrying tetracycline-resistant *Eschericia coli* in a married couple, one of whom was receiving antibiotics. *Antimicrob. Agents Chemother.*, **16**, 225-230.

PETROCEILOU, V., GRINSTED, J., RICHMOND, M.H. (1976). R-plasmid transfer *in vivo* in the absence of antibiotic selection pressure. *Antimicrob. Agents Chemother.*, **10**, 753-761.

POE, M. (1976). Antibacterial synergism: A proposal for chemotherapeutic potentiation between trimethoprim and sulphamethoxazole. *Science*, **174**, 533-535.

QUMSIEH, M.J., YOUNG, H-K. (1991). Cloning of the type Ib trimethoprim-resistant dihydrofolate reductase gene and preparation of a specific biotinylated DNA probe. *J. Antimicrob. Chemother.*, **27**, 707-712.

REVES, R.R., FONG, M., PICKERING, L.K., *et al* (1990). Risk factors for fecal colonization with trimethoprim-resistant and multiresistant *Eschericia coli* among children in day-care centres in Houston, Texas. *Antimicrob. Agents Chemother.*, **34**, 1429-1434.

REVES, R.R., MURRAY, B.E., PICKERING, L.K., et al (1987). Children with trimethoprim and ampicillin-resistant fecal *Eschericia coli* in day-care centres. *J. Infect. Dis.*, **156**, 758-762.

RICHARDS, H., DATTA, N. (1982). Plasmids and transposons acquired by *Salmonella typhi* in man. *Plasmid*, **8**, 9-14.

ROBERTS, W. (1874). Studies on Biogenesis. *Phil. Trans. Royal Soc. (Series B)*, **164**, 457-477.

ROTH, B., FALCO, E.A., HITCHINGS, G.H. (1962). 5-benzyl-2,4-diamino-pyrimidines as antibacterial agents. I synthesis and antibacterial activity *in vitro. J Med. Chem.* , **5**, 1103-1123.

ROUCH, D.A., MESSEROTTI, L.J., LOO, L.S.L., et al (1989). Trimethoprim resistance transposon Tn4003 from *Staphylococcus aureus* encodes genes for a dihydrofolate reductase and thymidylate synthetase flanked by three copies of IS257. *Mol. Microbiol.*, **3**, 161-175.

SAMBROOK, J., FRITSCH, E.F., MANIATIS, T. (1989). Molecular cloning: A laboratory manual (2nd edition). Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York.

SCHMIDT, F.R.J., NÜCKEN, E.J., HUNSCHKE, R.B. (1989). Structure and function of hotspots providing signals for site-directed specific recombination. *Mol. Gen. Genet.*, **2**, 709-717.

SCHMITT, R. (1986). Molecular biology of transposable elements. *J. Antimicrob. Chemother.*, **18**(Suppl. C), 25-34.

SCUDAMORE, R.A., GOLDER, M. (1989). Limited contribution of outer-membrane penetration barrier towards intrinsic antibiotic resistance of *Pseudomonas aeruginosa*. *Can. J. Microbiol.*, **28**, 169-175.

SHAHID, N.S., RAHAMAN, M.M., HAIDER, K. *et al* (1985). Changing pattern of resistant Shiga bacillus (*Shigella dysenteriae* type I) and *Shigella flexneri* in Bangladesh. *J. Infect. Dis.*, **152**, 1114-1119.

SHAPIRO, R.O. (1979). Molecular model for the transposition of bacteriophage mu and other transposable elements. *Proc. Nat. Acad. Sci.*, **76**, 1933-37.

SHAPIRO, R.O., SPORN, P. (1977). Transposon Tn402 a new transposable element determining resistance that inserts in bacteriophage lambda. *J. Bacteriol.*, **129**, 1632-1635.

SHEARS, P., SALIMAN, G., HART, C.A. (1988). Occurrence of multiple resistance and R-plasmid in *Enterobacteriacae* isolated from children in the Sudan. *Epidemiol. Infect.*, **100**, 73-81.

SHELDON, R. (1977). Altered dihydrofolate reductase in *fol* regulatory mutants of *Escherichia coli* K12. *Mol. Gen. Genet.*, **151**, 215-219.

SHELDON, R., BREMNER, S. (1976). Regulatory mutants of dihydrofolate reductase in *Escherichia coli* K12. *Mol. Gen. Genet.*, **147**, 91-97.

SIBLER, R., MANSOURI, A. (1971). Regulation of folate dependant enzymes. *Ann. N. Y. Acad. Sci.*, **186**, 55-69.

SIITONEN, A. (1992). *Escherichia coli* in faecal flora of healthy adults: Serotypes P and type 1C fimbriae. non-P mannose resistant adhesins and hemolytic activity. *J. Infect. Dis.*, **166**, 1058-1065.

SINGH, K.V., REVES, R., PICKERING, L.K., MURRAY, B.E. (1992). Identification by DNA sequence analysis of a new plasmid-encoded trimethoprim resistance gene in fecal *Escherichia coli* isolates from children in day-care centres. *Antimicrob. Agents Chemother.*, **36**, 1720-1726.

SKÖLD, O., WIDH, A. (1974). A new dihydrofolate reductase with low trimethoprim sensitivity induced by an R-factor mediating high resistance to trimethoprim. *J. Biol. Chem.*, **27**, 933-937.

SMITH, H.W. (1976). Mutants of *Klebsiella pneumoniae* resistant to several antibiotics. *Nature*, **259**, 307-308.

SMITH, D.R., CALVO, J.M. (1980). Nucleotide sequence of the *Escherichia coli* gene coding for dihydrofolate reductase. *Nuc. Acids Res.*, **8**, 2255-2274.

SMITH, D.R., ROOD, L.J., BIRD, P.I., *et al* (1982). Amplification and modification of dihydrofolate reductase in *Escherichia coli*. Nucleotide sequence of *fol* genes from mutationally altered plasmids. *J. Biol. Chem.*, **257**, 9043-9048.

SOLÓRZANO, F., LEAÑOS, M., GUISCARFÉ, H. (1987). Resistancia antimicrobiana actual de *Salmonella typhi, Salmonella enteriditis* y *Shigella* spp. *Bol. Med. Hosp. Infant. Mexico*, **44**, 448-455.

STACEY, K.A., SIMSON, E. (1965). Improved method for the isolation of thymine requiring mutants of *Escherichia coli*. *J. Bacteriol.*, **90**, 554-555.

STEEN, R., SKÖLD, O. (1985). Plasmid borne or chromosomally mediated resistance by Tn*7* is the most common response to the ubiquitous use of trimethoprim. *Antimicrob. Agents Chemother.*, **27**, 933-937.

STOKES , H.W., HALL, R.W. (1989). A novel family of potentially mobile DNA elements encoding site-specific gene-integration: Integrons. *Mol. Microbiol.*, **3**, 1669-1683.

STONE, D., SMITH, S.L. (1979). The amino acid sequence of the trimethoprim-resistant dihydrofolate reductase specified in *Escherichia coli* R67. *J. Biol. Chem.*, **254**, 10875-10861.

SUNDERAN, B.P., MURTHY, K.V. (1984). Transferable plasmid-mediated drug resistance among non-O1 *Vibrio cholerae* and rough strains of *Vibrio cholerae* from Tamil Nadu, India. *J. Hyg.*, **92**, 59-65.

SUNDSTRÖM, L. (1989). The genes of trimethoprim resistance and their recombinational dissemination. *Acta Pharmaceutica Nordica*, **1**, 375.

SUNDSTRÖM, L., RADSTRÖM, P., SWEDBERG, G., SKÖLD, O. (1988). Site-specific recombination promotes linkage between trimethoprim and sulphonamide resistance genes. Sequence characterization of *dhfr*V and *sul*I and the recombination active locus of Tn*21*. *Mol. Gen. Genet.*, **213**, 191-201.

SUNDSTRÖM, L., ROY, P.H., SKÖLD, O. (1991). Site-specific insertion of three structural gene cassettes in transposon Tn*7*. *J. Bacteriol.*, **173**, 3025-3028.

SUNDSTRÖM, L., SKÖLD, O. (1990). The *dhfr*I trimethoprim resistance gene of Tn*7* can be found at specific sites in other genetic elements. *Antimicrob. Agents Chemother.*, **34**, 642-650.

SUNDSTRÖM, L., SWEDBERG, G., SKÖLD, O. (1993). Characterization of transposon Tn*5086*, carrying the site-specifically inserted gene *dhfr*VII mediating trimethoprim resistance. *J. Bacteriol.*, **175**, 1796-1805.

SUNDSTRÖM, L.,VINAYAGAMOORTHY, T., SKÖLD, O. (1987). Novel type of plasmid borne resistance to trimethoprim. *Antimicrob. Agents Chemother.*, **31**, 60-66.

SWIFT, G. McCARTHY, B.J., HEFFRON, F. (1981). DNA sequence of a plasmid-encoded dihydrofolate reductase. *Mol. Gen. Genet.*, **181**, 441-447.

SYKES, R.B., MATTHEWS, M. (1976). The β-lactamases of gram-negative bacteria and their role in resistance to β-lactam antibiotics. *J. Antimicrob. Chemother.*, **2**, 115-157

SYKES, R.B., SMITH, J.T. (1979). Biochemical aspects of β-lactamases of gram-negative organisms. In β-LACTAMASES (J.M.T. Hamilton-Miller and J.T. Smith eds) Academic press, London.

TABTIENG, R., WATTANASRI, S., ECHEVERRIA, P., *et al* (1989). An epidemic of *Vibrio cholerae* el tor inaba resistant to several antibiotics with a conjugative group C plasmid coding for type II dihydrofolate reductase in Thailand. *Am. J. Trop. Med. Hyg.*, **41**, 680-686.

TAIT, S., AMYES, S.G.B. (1989). The type S1 trimethoprim-resistant dihydrofolate reductase in *Staphylococcus aureus*. *J. Appl. Bact.*, **67**, xiv-xv.

TAKAHASHI, S., NAGANO, Y. (1984). Rapid procedure for the isolation of plasmid DNA and application to epidemiological analysis. *J. Clin. Micro.*, **20**, 608-613.

TAUXE, R.V., CAVANAGH, T.R., COHEN, M.L. (1989). Interspecies gene transfer *in vivo* producing an outbreak of multiply resistant shigellosis. *J. Infect. Dis.*, **160**, 1067-1070.

TAUXE, R.V., PUHR, N.D., WELLS, J.G., *et al* (1990). Antimicrobial resistance of *Shigella* isolates in the U.S: Importance of international travellers. *J. Infect. Dis.*, **162**, 1107-1111.

TAYLOR, D.E., CHUMPITAZ, J.C., GOLDSTEIN, F. (1985). Variability of Inc HI1 plasmids from *Salmonella typhi* with special reference to Peruvian plasmids encoding resistance to trimethoprim and other antibiotics. *Antimicrob. Agents Chemother.*, **28**, 452-455.

TENNHAMMER-EKMAN, B., SUNDSTRÖM, L., SKÖLD, O. (1986). New observations regarding the evolution of trimethoprim resistance. *J. Antimicrob. Chemother.*, **18**(Suppl. C), 67-76.

THEN, R., ANGEHRN, P. (1973). Nature of the bactericidal action of sulphonamides and trimethoprim, alone and in combination. *J. Infect. Dis.*, **162**(Suppl. 5), S498-S501.

THOMSON, C.J. (1990). Ph. D. Thesis. University of Edinburgh.

THOMSON, C.J., AMYES, S.G.B. (1993). Selection of variants of the TEM-1 β -lactamase, encoded by a plasmid of clinical origin, with increased resistance to β -lactamase inhibitors. *J. Antimicrob. Chemother.*, **31**, 655-664.

THOMSON, C.J., BARG, N.L., AMYES, S.G.B. (1990). N-terminal amino acid sequence and sub-unit structure of the novel type IIIb trimethoprim-resistant dihydrofolate reductase from *Shigella sonnei. J. Gen. Microbiol.*, **136**, 673-677.

THOMSON, C.J., TOWNER, K.J., CARTER, G.I., YOUNG, H-K., AMYES, S.G.B. (1990). Identification and cloning of the type IIIa plasmid-encoded dihydrofolate reductase gene from gram-negative bacteria in Britain. *J. Med. Microbiol.*, **31**, 213-218.

THOMSON, C.J., YOUNG, H-K., AMYES, S.G.B. (1990). N-terminal amino acid sequence and sub-unit structure of the type IV trimethoprim-resistant plasmid-encoded dihydrofolate reductase. *J. Med. Microbiol.*, **32**, 153-158,

THOMSON, C.J., YOUNG, H-K., AMYES, S.G.B. (1993). The role of thymine starvation in the expression of the type IV plasmid-encoded trimethoprim-resistant dihydrofolate reductase. *J. Med. Microbiol.*, **38**, 250-255.

THRELFALL, E.J., FROST, J.A., KING, H.C., ROWE, B. (1983). Plasmid-encoded trimethoprim-resistance in *Salmonella* isolated in Britain between 1970 and 1981. *J. Hyg.*, **90**, 55-60.

THRELFALL, E.J., ROWE, B., HUQ, I. (1980a). Plasmid-encoded multiple antibiotic resistance in *Vibrio cholerae* el tor from Bangladesh. *Lancet*, II, 1247-1248.

THRELFALL, E.J., WARD, L.R., ASHLEY., ROWE, B. (1980b). Plasmid-encoded trimethoprim-resistance in multiresistant *Salmonella typhimurium* isolated phage types 204 and 193 in Britain. *Br. Med. J.*, **280**, 1210-1211.

THRELFALL, E.J., WARD, L.R., ROWE, B. (1986). R-plasmids of *Salmonella typhimurium* in the United Kingdom. *J. Antimicrob. Chemother.*, **18**(Suppl. C), 175-176.

TIETZE, E., BREVET, J., TSCHÄPE, H. (1987). Relationships among the streptothricin resistance transposons Tn1825 and Tn1826 and the trimethoprim-resistance transposon Tn*7*. *Plasmid*, **18**, 246-249.

TOWNER, K.J. (1982). Resistance to trimethoprim among urinary tract isolates in the United Kingdom. *Rev. Infect. Dis.*, **4**, 456-460.

TOWNER, K.J. (1990). DNA probes for trimethoprim-resistant dihydrofolate reductases. *J. Med. Microbiol.*, **31**, 9-11.

TOWNER, K.J., CARTER, G.I. (1990). Cloning of the type VII trimethoprim-resistant dihydrofolate reductase and identification of a specific DNA probe. *FEMS. Microbiol. Lett.*, **58**, 19-22.

TOWNER, K.J., CARTER, G.I., YOUNG, H-K., AMYES, S.G.B. (1991). Detection of novel trimethoprim resistance determinants in the United Kingdom using biotin-labelled DNA probes. *Epidemiol. Infect.*, **106**, 63-70.

TOWNER, K.J., PEARSON, N.J., PINN, P.A., O'GRADY, F. (1980). Increasing importance of plasmid-mediated resistance in Enterobacteria: Two six month clinical surveys. *Br. Med. J.*, **280**, 517-519.

TOWNER, K.J., PINN, P.A. (1981). A transferable plasmid conferring only a moderate level of resistance to trimethoprim. *FEMS. Microbiol. Lett.*, **10**, 271-272.

TOWNER, K.J., SLACK, R.C.B. (1986). Effect of changing selection pressures on trimethoprim resistance in *Enterobacteriaceae*. *Eur. J. Clin. Microbiol.*, **5**, 502-506

TOWNER, K.J., WISE, P.J., LEWIS, M.J. (1986). Relationships between trimethoprim R-plasmids obtained from human and animal sources. *J. Appl. Bacteriol.*, **61**, 535-540.

TOWNER, K.J., YOUNG, H-K., AMYES, S.G.B. (1988). Biotinylated DNA probes for trimethoprim-resistant dihydrofolate reductases type IV and V. *J. Antimicrob. Chemother.*, **22**, 285-291.

TOWNER, K.J., YOUNG, H-K., THOMSON, C.J., AMYES, S.G.B. (1990). Detection in the United Kingdom of trimethoprim-resistant *Escherichia coli* encoding the type V dihydrofolate reductase. *Eur. J. Clin. Microbiol.*, **9**, 149-150.

TRÉFOUEL, J., NITTI, F., BOVET, D. (1935). Activité du *p*-aminophénylsulfamide sur les infections streptococciques éxperimentales de la souris et du lapin. *C. R. Soc. Biol. Paris,* **120**, 756-758.

TSAKRIS, A., JOHNSON, A.P., LEGAKIS, N.J., TZOUVELEKIS, L.S. (1993). Prevalence of type I and type II DHFR genes in trimethoprim resistant urinary isolates of *Escherichia coli* from Greece. *J. Antimicrobial. Chemother.*, **31**, 665-671.

TSAKRIS, A., VATOPOULOS, A.E., JOHNSON, A.P., *et al* (1992). Prevalence of a plasmid-mediated type II *dhfr* gene among trimethoprim-resistant urinary pathogens in Greek hospitals. *J. Antimicrob. Chemother.*, **29**, 405-415.

URBINA, R., PRADO, V., CANELO, E. (1989). Trimethoprim resistance in Enterobacteria isolated in Chile. *J. Antimicrob. Chemother.*, **23**, 143-149.

VAN, R., MORROW, A.L., REVES, R.R., PICKERING, L.K. (1991). Environmental contamination in child day-care centres. *Am. J. Epidemiol.*, **133**, 460-470.

VOOGD, C.E., SCHOT, C.S., VAN LEEUWEN, W.J., VAN KLINGEREN, B. (1992). Monitoring of antibiotic resistance in *shigellae* isolated in the Netherlands 1984-1989. *Eur. J. Clin. Microbiol. Infect. Dis.*, **11**, 164-167.

WADDELL, W.J. (1956). A simple ultraviolet spectrophotometric method for the determination of protein. *J. Lab. Clin. Med.*, **48**, 311-314.

WATANABE, T., FUKUSAWA, T. (1960). "Resistance transfer factor" an episome in *Enterobacteriaceae. Biochem. Biophys. Res. Commun.*, **3**, 660-665.

WERNER, R.G., GOETH, H. (1984). Trimethoprim, failure to penetrate into *Pseudomonas aeruginosa* cells. *FEMS. Microbiol. Lett.*, **23**, 201-204.

WIEDEMANN, B., MEYER, J.F., ZUHLSDORF, M.T. (1986). Insertion of resistance genes into Tn*21*-like transposons. *J. Antimicrob. Chemother.*, **18**(Suppl. C), 85-92.

WILKINSON, P.J., REEVES, D.S. (1979). Tissue penetration of trimethoprim and sulphonamides. *J. Antimicrob. Chemother.*, **5**(Suppl. B), 159-168.

WOHILEBEN, W., ARNOLD, W., BISSONNETTE, L., *et al* (1989). On the evolution of the Tn*21*-like transposons: Sequence analysis of the gene (*aac*1) for the gentamicin acetyltransferase-3-1 [AAC(3)-1] another member of the Tn21-based expression cassette. *Mol. Gen. Genet.*, **217**, 202-208.

WOODS, D.D. (1940). The relation of *p*-aminobenzoic acid to the mechanism of action of sulphonamide. *Br. J. Expt. Path.*, **21**, 74-90.

WYLIE, B.A., AMYES, S.G.B., YOUNG, H-K., KOORNHOF, H.J. (1988). Identification of a novel plasmid-encoded dihydrofolate reductase mediating high level resistance to trimethoprim. *J. Antimicrob. Chemother.*, **22**, 429-435.

WYLIE, B.A., KOORNHOF, H.J. (1991). Nucleotide sequence of dihydrofolate reductase type VI. *J. Med. Microbiol.*, **35**, 214-218.

YANISCH-PERRON, C., VIERA, J., MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: Nucleotide sequences of M13mp18 and pUC19 vectors. *Gene*, **33**, 103-119.

YOUNG, H-K., AMYES, S.G.B. (1985). Characterization of a new transposon-mediated trimethoprim-resistant dihydrofolate reductase. *Biochem. Pharmacol.*, **34**, 4334-4337.

YOUNG, H-K., AMYES, S.G.B. (1986a). A new mechanism of plasmid trimethoprim resistance. Characterization of an inducible dihydrofolate reductase. *J. Biol. Chem.*, **261**, 2503-2505.

YOUNG, H-K., AMYES, S.G.B. (1986b). Plasmid trimethoprim resistance in *Vibrio cholerae*; Migration of the type I dihydrofolate reducase gene out of the *Enterobacteriaceae*. *J. Antimicrob. Chemother.*, **17**, 697-703.

YOUNG, H-K., JESUDASON, M.V. (1990). Antibiotic resistant bacteria in drinking water in south India. *J. Pharmacol.*, **42**, 66p.

YOUNG, H-K., JESUDASON, M.V., KOSHI, G. AMYES, S.G.B. (1986a). Trimethoprim resistance among urinary pathogens in south India. *J. Antimicrob. Chemother.*, **17**, 615-621.

YOUNG, H-K., JESUDASON, M.V., KOSHI, G. AMYES, S.G.B. (1986b). Unusual expression of new low-level trimethoprim-resistance plasmids. *J. Clin. Microbiol.*, **24**, 61-64.

YOUNG, H-K., SKURRAY, R.A., AMYES, S.G.B. (1987) Plasmid mediated trimethoprim resistance in *Staphylococcus aureus*: Characterization of the first gram-positive dihydrofolate reductase (S1). *Biochem. J.* **243**, 309-312.

YOUNG, H-K., THOMSON, C.J., AMYES, S.G.B. (1993). The induction of trimethoprim resistance encoded by the type IV dihydrofolate reductase. *J. Med. Microbiol.*, **38**, 256-261.

ZAMAN, K., YUNIS, M.D., BAQUI, A.H., *et al* (1983). Co-trimoxazole resistant *Shigella dysenteriae* type I outbreak in a family in rural Bangladesh. *Lancet*, **II**, 796-797.

ZOLG, J.W., HÄNGII, U.J. (1978). Isolation of a small DNA fragment carrying the gene for a dihydrofolate reductase from a trimethoprim resistance factor. *Mol. Gen. Genet.*, **9**, 697-710.

ZUHLSDORF, M.T., WEIDEMANN, B. (1992). Tn*21*-specific structures in gram-negative bacteria from clinical isolates. *Antimicrob. Agents Chemother.*, **36**, 1915-1921